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ANTIGENIC VARIATION IN VIRULENCE
DETERMINANTS OF *STREPTOCOCCUS*
ZOOEPIDEMICUS* AND *ACTINOBACILLUS EQUULI
INVOLVED IN LOWER AIRWAY DISEASE OF THE
HORSE AND STRATEGIES TOWARDS
PROTECTIVE IMMUNISATION

BY

CHANTELLE LOUISE WARD, BSc

A thesis submitted in partial fulfilment of the requirements
of the Open University for the degree of
Doctor of Philosophy,
pertaining to the discipline of microbiology

December 1997

Animal Health Trust
P.O. Box 5
Newmarket
Suffolk
CB8 7DW

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ABSTRACT

Lower airway disease (LAD) of the training Thoroughbred horse is characterised by poor performance and excess mucus and pus in the trachea. This condition, although often referred to as 'the virus', is significantly associated with *Streptococcus zooepidemicus* and *Actinobacillus/Pasteurella* species without evidence of viral infection.

The degree of antigenic variability in these bacteria was assessed, to judge the number of strains that might be required in future vaccines. Particular attention was paid to the putative virulence determinants of the M-like protein and capsule of *S. zooepidemicus* and the iron regulated proteins (IRPs), produced only in low available iron concentrations, of *Actinobacillus equuli*.

A range of *Actinobacillus/Pasteurella* species were isolated from the lower airway of horses, but more than half were accounted for by *A. equuli*. *A. equuli* bound and utilised equine holotransferrin for growth. The constitutive proteins of *A. equuli* were antigenically cross-reactive, but IRPs were more antigenically varied. Vaccines based on interference of IRPs would be likely to need more than one strain of *A. equuli*.

Hot acid extracts of *S. zooepidemicus* were antigenically cross-reactive, but there was less opsonogenic cross-reactivity in horse blood containing natural antibodies than rabbit antisera to extracts. A vaccine based on M-like protein would be likely to need more than one type of *S. zooepidemicus*, but no relationship could be found between ribotype / antigenic profile and opsonogenic behaviour, suggesting that M-like protein is not necessarily the principal anti-phagocytic mechanism for these isolates, at least *in vitro*.

S. zooepidemicus produces a hyaluronate capsule thought to be a virulence determinant, which is tightly bound by a protein (HAP). Immunisation with recombinant HAP significantly increased survival time and clearance of bacteria in mice challenged intraperitoneally with *S. zooepidemicus*. Recombinant HAP may have acted as a surrogate antigen for the capsule.

Introduction of bacteria into ponies intratracheally produced a dose response for *S. zooepidemicus* and *A. equuli*. However, so much intercurrent infection developed (due to *S. zooepidemicus* and *Bordetella bronchiseptica*) it was concluded that conventional ponies would be unsuitable for straightforward studies of vaccine efficacy in response to experimental challenge. Alternative ways forward are discussed.

SUMMARY

Lower airway disease (LAD) of the training Thoroughbred horse is characterized by poor performance and excess mucus and pus in the trachea. This condition, although frequently referred to as “the virus”, is significantly associated with *Streptococcus zooepidemicus* and *Actinobacillus/Pasteurella* species without evidence of viral infection.

The objective was to assess the degree of antigenic variability in these bacteria to judge the number of strains that might be required in future vaccines. Particular attention was paid to the putative virulence determinants of the M-like protein and capsule of *S. zooepidemicus* and the iron regulated proteins (IRPs), produced only in low available iron concentrations, of *Actinobacillus equuli*.

More than half (52.1%) of the tracheal wash isolates originally characterized as *Actinobacillus/Pasteurella* species involved were identified as *A. equuli*, 18.3% were *Actinobacillus suis* -like, 12.7% were *Pasteurella pneumotropica*, 8.5% were *Actinobacillus ligniersii*, 7.0% were *Pasteurella haemolytica* and 5.6% were *P. mairii*, suggesting that a range of *Actinobacillus/Pasteurella* species can be isolated from the lower airway of horses, but more than half are accounted for by *A. equuli*.

All isolates cultured from cases of LAD were selected on the basis of their temporal and geographical separation. In iron limited media, all isolates bound and utilised equine holotransferrin for growth, but not iron bound to rabbit transferrin, human transferrin, or a number of other equine iron carrying proteins. The constitutive proteins of *A. equuli* were antigenically cross-reactive, but IRPs were more antigenically varied. Vaccines based on interference of IRPs would be likely to need more than one strain of *A. equuli*.

Hot acid extracts of *S. zooepidemicus* were antigenically cross-reactive, but there was less opsonogenic cross-reactivity in horse blood containing natural antibodies than rabbit antisera to extracts. A vaccine based on M-like protein would be likely to need more than one type of *S. zooepidemicus*, but no relationship could be found between ribotype /

antigenic profile and opsonogenic behaviour, suggesting that M-like protein is not necessarily the principal anti-phagocytic mechanism for these isolates, at least *in vitro*.

S. zooepidemicus produces a hyaluronate capsule thought to be a virulence determinant, which is tightly bound by a protein (HAP). Immunisation with recombinant HAP significantly increased survival time and clearance of bacteria in mice challenged intraperitoneally with *S. zooepidemicus*. Recombinant HAP may have acted as a surrogate antigen for the capsule.

Ponies were selected for challenge at the place of supply on the basis of their limited serum antibody to IRPs or M-like proteins for the strains under study. After transport to Home Office designated premises and following introduction of 10^{10} , 10^9 and 10^8 cfu intratracheally, a dose response for *S. zooepidemicus* and *A. equuli* was observed in severity of clinical observations and gross pathology at post mortem examination, 3 or 4 days after infection. However, so much intercurrent infection developed (due to *S. zooepidemicus* and *Bordetella bronchiseptica*) it was concluded that conventional ponies would be unsuitable for straightforward studies of vaccine efficacy in response to experimental challenge. Alternative ways forward are discussed.

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1.0 CHAPTER ONE

PREFACE, INTRODUCTION AND OBJECTIVES

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1.2 PREFACE TO THE INTRODUCTION

The anatomy, physiology, cytology, defence mechanisms and bacterial flora will be outlined in the healthy equine respiratory tract. The diagnosis and aetiology of airway disease in the equine will be reviewed, then a more detailed review of bacteria and vaccination will be made. Throughout the introduction, comparisons with other respiratory diseases in other species will be drawn and differences contrasted where they might throw light on the equine disease. Respiratory disease in the horse and particularly the discovery that *Streptococcus zooepidemicus* and *Actinobacillus/Pasteurella* are associated lower respiratory tract infections (LRTIs) will be examined in detail. The biology of *S. zooepidemicus* and *Actinobacillus/Pasteurella* infections and their involvement in other equine and mammalian diseases will be reviewed. The vaccination strategies used for other infectious agents will be assessed as they might relate to equine bacterial LRTIs. Gaps in our knowledge of *S. zooepidemicus* and *Actinobacillus/Pasteurella* in relation to immunity in the horse will be identified to help prepare the objectives of the study.

1.3 INTRODUCTION

1.3.1 The equine respiratory tract - in health

1.3.1.1 Anatomy and physiology

The lungs and the passages through which air passes to and from the lungs comprise the respiratory tract. The main function of the lungs are to supply inspired air containing oxygen, for metabolism by the tissues and to remove metabolic carbon dioxide via the bloodstream (Derksen, 1991).

The nose is divided internally into two vestibules (where the nasal mucosa begins), leading to the larger nasal cavity from which diverticula called paranasal sinuses extend. The nasal cavity is divided by the nasal septum and mucosa covered turbinate bones (conchae) protrude from the dorsal and lateral walls into the cavity interior, which warm, humidify and filter the inspired air. The lower compartment of the equine pharynx is divided into the oropharynx, which extends to the epiglottis and is surrounded by tonsillar tissue, and the laryngopharynx, which narrows down to the oesophagus. The laryngopharynx connects with the tracheobronchial tree. The laryngeal mucous membrane contains many mucous glands and lymphoid aggregations. The trachea and bronchi form a continuous tube held open by rings of cartilage, which connects the larynx with the lungs. The trachea extends from the larynx and bifurcates above the heart. It lies close to major blood vessels and their tributaries, the oesophagus and mediastinal lymph nodes. The structure of the large bronchi are very similar to the trachea but on smaller bronchi, the cartilage rings are gradually replaced by irregular plaques, eventually becoming bronchioli, which are small enough to be kept expanded by air pressure in the respiratory tree. The left lung has cranial and caudal lobes, and partly due to the position of the heart, is slightly thinner than the right lung in the horse, which also has an accessory lobe at its base. The pulmonary artery and pulmonary vein combine with the chief bronchus, and division of the artery corresponds to the bronchi. Lymph associated with the equine airway drains to small pulmonary nodes, then to larger tracheobronchial lymph nodes (Dyce *et al.* 1996).

The bulk movement of air into and out of the respiratory tract, or ventilation (Murray, 1986), is matched with metabolic rate by the respiratory centres in the brain stem, which uses receptors to coordinate the muscles controlling breathing (Berger, Mitchell & Severinghouse, 1977). Breathing is achieved by the movement of the diaphragm and intercostal muscles, which exert a driving pressure to overcome pulmonary elastic recoil, airflow resistance and inertial forces such that air enters the lung. Exhalation is passive in most animals, but the horse uses abdominal muscles to further decrease lung volume, once residual capacity is reached.

The volumes of air involved in breathing are less than the tidal volume of the lungs because as much as 70% in the horse at rest is used to ventilate the conducting airways, leaving approximately 30% for ventilation of the alveoli (Derksen *et al.* 1982). Much of the airflow resistance is due to the upper airway (Robinson & Sorensen, 1978). Exercising horses increase the airflow through the upper airway from approximately 4L/sec at rest to more than 75L/sec (Derksen, 1991). Cardiac output and pulmonary artery pressure also increases markedly in the exercising horse; the pulmonary circulation comprises an extensive capillary network, which delivers blood to the alveoli for gaseous exchange (Fung & Sowbin, 1969). Gravity ensures that the dorsal region receives less blood than the ventral lung mass (Amis, Pascoe & Hornof, 1984) and the air pressure in the dorsal lung region of the horse is sub-atmospheric (Derksen & Robinson, 1980) resulting in preferential ventilation of the ventral regions.

1.3.1.2 Airway defence mechanisms

The single layer of epithelial cells is needed for gaseous exchange between inspired air and the bloodstream, the surface is more susceptible to allergens, infectious, chemical and physical agents than the hard, dry multilayered skin covering most of the body surface. The horse lung for example, has more than 2000 m² of surface exposed to the external environment to allow enough gaseous exchange surface for metabolic needs (Gehr *et al.*

1981). These surfaces are protected by both mechanical and immunological defence mechanisms.

The turbinates inside the nasal cavity cause turbulence that may direct aerosol particles onto their mucus covered walls, where they can stick. Mucus comprises a viscoelastic gel layer mainly of associated glycoproteins and proteoglycans, which traps particles, and a sol layer which also contains lipids (Coles *et al.* 1984). The serous sol layer is produced in the terminal bronchioles and alveoli, probably in part by Clara cells (Widdicombe and Pack, 1982). Goblet cells in the bronchioles, trachea and upper respiratory tract (URT) secrete the viscous gel layer over the sol layer, which is protected from dessication. The gel layer is continuous in the central airway, whilst rafts may float on the sol layer in the distal airways (Iravani & Melville, 1976). If the sol layer is thickened, clearance of mucus is retarded (Dalhamn, 1956), so airway fluid balance, regulated by sodium chloride absorption and secretion, is probably crucial to health (Frizzel, 1988).

Ciliated cells, mucus secreting cells, sub-epithelial uni- and multicellular mucus glands with associated myoepithelial cells, line most of the upper respiratory tract and the tracheal wall. Mechanical stimulation is thought to control mucus production (Sleigh, Blake & Lison, 1988). Increased mucus secretion is caused by parasympathetic stimulation (Nadel, 1981), which in turn stimulates ciliary motility (Satin & Derksen, 1985). Myoepithelial cells aid the movement of secretions into the airway from secretory cells (Nadel, Davis & Phipps, 1979), to provide a protective mucous covering which is moved towards the pharynx by cilia, which coordinate their action with cilia on adjacent cells. Cilia are microtubular structures of 5-6 μ m long, with 3-7 25-35 nm long claws at the tip; there are approximately 200 cilia per cell. Cilia are powered by hydrolysis of ATP, which enables an outer microtubule to slide along the inner microtubule of an adjacent pair, which when coordinated with other adjacent pairs, results in ciliary bending (Greenstone and Cole, 1985; Satir, 1974). Particles can be trapped in the mucus, which is transported by cilia to the pharynx and then swallowed (Sleigh, Blake & Lison, 1988). Cilia propel fluids of certain composition by asymmetric cyclic movements, where when fully extended, the tip

passes through the sol layer and contacts the gel layer above. In human infants, it has been suggested that the immature ciliary apparatus leads to inefficient removal of bacteria and inflammatory debris (Koterba, 1991), and impaired ciliary motion is thought to predispose adult humans (Afzelius, 1979), and dogs (Killingsworth, Slocombe & Wilsman, 1987) to infection.

Airborne particles smaller than $0.5\mu\text{m}$ tend to remain in suspension so are exhaled (Brain & Valberg, 1979). If particles manage to bypass the mechanical mucociliary host defences (typically $0.5\text{-}10\mu\text{m}$ in size), they can be phagocytosed by alveolar macrophages, then digested, or macrophages can migrate to the mucociliary escalator once particles have been successfully ingested, where they are transported to the pharynx and are eliminated, (Brain, 1985). Some penetrate alveolar epithelia and are removed by the lymphatic system. Macrophages have a long life in lung tissue, are capable of synthesizing large quantities of enzymes and show chemotactic behaviour. Lysosomal enzymes and an oxygen dependent antimicrobial system are the killing mechanisms, although some macrophages produce reactive nitrogen intermediates for which the functions are uncertain. The cell surface has Fc and complement receptors to enable attachment to opsonised particles. Neutrophils are also chemotactic and have the ability to move through tissues to sites of infection, where they phagocytose opsonised particles very rapidly, but they are short lived. They contain different lysosomal enzymes to macrophages and an equivalent but different oxygen-dependant antimicrobial system. Eosinophils are less effective at phagocytosis and killing microbes generally, but are effective at removing immune complexes and killing parasites. Their granules distinctly contain inflammatory mediator blockers. T lymphocytes differentiate to release lymphokines in the course of the cell mediated response, whilst B lymphocytes are stimulated to differentiate into antibody secreting cells.

Immunological surface protective mechanisms against bacteria that give resistance to disease primarily involve antibody and complement. For example, convalescent sera of lambs challenged with *P. haemolytica* (Sutherland, 1988) show complement dependent antibody mediated killing. Specific antibody can be generated by prior exposure to antigen.

If the animal has mucosal memory cells from prior exposure to antigen, many will be stimulated to produce antibody producing a greater response with a shorter time lag and minimizing host cell damage. Even if antibody levels are undetectable, there can be enough mucosal memory cells to generate an accelerated response. Secretory IgA is produced by B lymphocytes distributed throughout the lung and the walls of the airway in response to local antigenic stimulation. The predominant antibody isotype in the equine upper respiratory tract is secretory IgA, whereas the bronchioles and alveoli contain a large proportion of IgG (Mair, Stokes and Bourne, 1987), which is probably secreted through tight junctions in the airway epithelia. There are intermediate concentrations of these isotypes in the trachea and in serum. The main function of secretory IgA present in mucus is to block adherence of bacteria and viruses to the respiratory epithelia, although it can neutralize viruses and some viral and bacterial enzymes or toxins. IgG is formed in the body for many years after exposure to antigen and opsonises particles, which leads to enhanced phagocytosis and removal (Mimms *et al*, 1995); it is probably only important once inflammation and transudation of serum occur (Tizard, 1992). Whilst older animals are protected by antibody raised by their prior exposure to antigen (active immunity), neonatal foals are only protected by passive immune transference of IgG from colostrum. The IgG passes into the circulation and tissues and peaks at 48 hours after birth, then declines according to a half life of approximately 23 days. Colostrum also contains lymphocytes and complement which may have a protective function (Rossdale, 1995).

1.3.1.3 Cytology and bacteriology

Alveolar macrophages, along with small numbers of lymphocytes, neutrophils and eosinophils, are present in the walls and air spaces of the healthy airway (Derksen *et al*. 1985), but a study of Thoroughbred horses in training suggested that macrophages and lymphocytes were the predominant cell types, and haemosiderophages were found in 90% of cases or more, the presence of which indicates pulmonary haemorrhage or previous presence of blood in the airways (Whitwell and Greet, 1984).

An autopsy study of Thoroughbred and non-Thoroughbred horses found that the lungs are normally sterile, or contain low numbers of bacteria which, in the absence of inflammation, are thought to be transient contaminants in the process of being removed by clearance mechanisms (Blunden and Mackintosh, 1991). Similar observations have been made for cattle (Collier and Rossow, 1964). A similar study of healthy horses found that only 8% contained organisms of known pathogenicity (Sweeney, Beech and Roby, 1985). These bacteria were present in small numbers, so it was suggested that in any study, cultures must be quantitated and assessed in relation to the presence of inflammation to determine the true significance of a bacterial presence (Mansmann, 1976).

1.3.2 The equine respiratory tract - in disease

1.3.2.1 Diagnosis

Inflammatory disease of the lower airway has clinical signs which vary in severity. These range from unexpectedly poor performance on training gallops or in races, to symptoms of pneumonia, or pleuropneumonia, which include pyrexia, tachypnea, depression, and varied amounts of coughing and mucopurulent discharge. Thickening of the mucosa around the entrance to the sinus system may obstruct its drainage, damming back catarrhal exudate (Dyce *et al.* 1996). During a fast gallop, the horse on average breathes in and out twice per second, so the slightest thickening of the trachea, or increase in exudate has the capacity to reduce performance enormously (Art and Lekeux, 1989). Bacterial lower airway disease (LAD) involves bacterial colonization of the lung, which can develop into pneumonia and / or pulmonary abscess formation and subsequent extension to the pleura, leading eventually to pleural effusion. Pneumonia is an inflammation of the lung, whilst pleuropneumonia also affects the pleural cavity. Severe infections such as pleuropneumonia are considered uncommon in the United Kingdom (Mair and Lane, 1989), but the more common sub-clinical infections are thought to be particularly important in disease transmission (Gordis, 1996).

Clinical signs are usually noticed as poor performance by the trainer, who calls a veterinary surgeon to carry out further investigation. This involves examination of the trachea by

endoscopy, and collection of a lavage sample via a catheter inserted down the biopsy channel, which is then used for routine bacteriological and cytological investigations (Whitwell and Greet, 1984; Morris, 1984; Sweeney *et al*, 1985,1989,1991). It is assumed that isolates cultured represent a mixture of tracheal and lung tissue flora (Hoffman *et al*. 1993). Tracheal lavage samples can also be collected by a technique known as transtracheal aspiration, which involves aseptic puncture of the trachea, but rare secondary complications can occur from transfer of infection upon withdrawal of the needle (Mansmann and Knight, 1972). Consequently, it is less commonly used, although its advantage is that it avoids contamination of the sample by nasopharyngeal organisms collected as the endoscope is inserted. The epithelial lining fluid and cells in the alveoli can be sampled using either an endoscope or blind tube in a technique known as bronchoalveolar lavage. Bacteria from foals can be most easily sampled using this technique, (Hoffman *et al*. 1993), but it is of limited use for diagnosis of non-diffuse lung disease, because only a specific area is lavaged and diseased areas stand a good chance of being missed. Consequently, tracheal lavage is believed to give a better indicator of the health of the whole lung (Beech, 1991). Cytology, along with evaluation of the amount of visible tracheal mucus, leads to an evaluation of the degree of lower airway inflammation (Wood and Chanter, 1994). Nasopharyngeal swabs can also be taken at the same time for virology investigation, but bacteriology of the nasopharynx is of limited use for lower airway disease diagnosis because bacterial species isolated in high numbers from tracheal washes are frequently not cultured in the nasopharynx (Wood and Chanter, 1994).

Ultrasonography, radiography and occasionally scintigraphic imaging techniques, lung biopsy, and thoracocentesis can also be used to aid the diagnosis of respiratory problems, although they are not a substitute for sampling for bacteriology, virology, or cytology. Radiology is the method of choice if the horse appears normal on auscultation but disease is suspected, as may be the case with poor performance syndrome and enables lesions deep in the pulmonary parenchyma to be seen, although only lateral films can be obtained. However, it is probably a technique confined to specialist centres, because the powerful

generator required to overcome the size and respiratory motion of the horse make equipment non-portable and expensive (Leyland and Baker, 1975). Ultrasonography is useful to characterize disease severity, adhesions, pleural thickening, necrosis, compression, or atelectasis (Sweeney and Maxson, 1995). Equipment is cheaper and portable, but it is the method of choice if the horse is abnormal on auscultation, such as might be heard with pleuropneumonia, because it is difficult to see lesions in the normally aerated lung (Reimer, Reef and Spencer, 1989). Thoroughbred racehorses with symptoms of poor performance typically have small consolidations along the ventral lung margins, visible on ultrasound (Rantanen, 1993). Thoracocentesis is indicated when there is evidence of pleural effusion, whilst lung biopsies are taken for diffuse interstitial lung disease cases, but the technique risks lacerating blood vessels (Raphael and Gunson, 1981).

Whilst these more technological diagnostic tools are helpful, bacteriology is essential as an adjunct to differentiate between bacterial lower airway disease and other diseases, such as allergic lung disease (Whitwell and Greet, 1984; Wood *et al*, 1993; Blunden *et al*. 1985).

1.3.2.2 Aetiology

A survey evaluating losses that occurred in the Thoroughbred industry in the UK and Ireland revealed that respiratory problems were a common cause of wastage (Jeffcott *et al*. 1982). Further study into why racehorses were unfit for training identified respiratory problems as the second largest cause of wasted training days (after lameness) in young Thoroughbred horses aged up to four years (Burrell *et al*. 1985). A more recent study of British Thoroughbred horses in training revealed that 27% of clinically normal horses had evidence of inflammatory lower airway disease (Sweeney *et al*, 1993). Although not so well documented, similar problems have been identified elsewhere. For instance, a survey of the Australian Thoroughbred industry indicated that infectious respiratory disease was the main cause of wastage and a scientific study is currently in progress (Equine Research News, 1996). Clinical respiratory disease is experienced in the yards of most trainers each year and is commonly referred to as "the cough" (Johnson, 1986). Animals with sub-clinical episodes of "poor performance syndrome" referred to "as having the virus",

describes unexpectedly poor performance during training gallops or in races, of many horses in one stable (Mumford and Rosedale, 1982).

The most well documented infectious agents of equine airway disease are viruses; these include equine influenza, equine herpes viruses (EHV-1- rhinopneumonitis, EHV-4 and EHV-2- cytomegalovirus), equine adenovirus, equine arteritis virus (EAV) and Picornaviruses (rhinovirus). An epidemic of equine influenza is generally considered to be devastating as it has high morbidity and is highly infectious, but attempts are made to vaccinate as many animals as possible against it, using a preparation which is regularly updated. EAV is also devastating as it can be venereally transmitted and cause abortion, in addition to being a respiratory pathogen (Timoney and McCollum, 1988), but until recently it was not found in the United Kingdom, and the one outbreak was successfully contained; there is no vaccine available. Adenovirus causes mild upper respiratory tract infections, which are especially common in foals and rhinovirus a mild infection which usually requires no treatment (Johnson, 1986; Beech, 1991). EHV has gradually increased in the UK since the 1970s, and although it does not cause epidemics, EHV-1 is a particular cause for concern, since it can cause neurological disorder, abortion, stillbirth and neonatal death; at present there is no effective vaccine. These viral infections can be diagnosed by serology and / or virus isolation and it was noticed by some that some equines had signs of respiratory disease uncomplicated by viruses.

Bacteria were found to be of greater significance than previously thought, as one study isolated beta haemolytic streptococci, *Pasteurella* spp. and *E. coli* from in horses with pneumonia or pleuropneumonia (Sweeney *et al.* 1991). Large numbers of a beta haemolytic streptococcus, identified as *Streptococcus zooepidemicus* in another study along with, *S. pneumoniae* and *Actinobacillus/ Pastuerella* species, were statistically associated with inflammatory lower airway disease in young training Thoroughbred horses (Wood *et al.* 1993). *S. zooepidemicus* had long been thought of as a cause of secondary infection following Influenza or Adenovirus infection of the respiratory tract, but in these cases, it was uncomplicated by the presence of viruses. Serial episodes of disease in the same group

of horses commonly involved these bacteria. Recent work confirmed the association of *S. zooepidemicus* with LAD in Thoroughbred horses, but showed that *S. pneumoniae* and *Pasteurella* species were less significantly associated with LAD than originally thought. It was calculated that Thoroughbred horses in training aged between two and three years old spend approximately 33% of their time in training with LAD, which could impair performance and has considerable welfare implications. Two year olds were found to be most affected, the implications of which are important for the racing industry since this is when horses have to prove their ability (Burrell *et al.* 1996). A study comparing the winnings of horses with a history of bacterial LAD to those with none suggested that beta haemolytic Streptococcal infections did influence performance (Bernard *et al.* 1991).

LAD most commonly manifests itself as poor performance because the horse at a fast gallop of 150 strides per minute takes less than half a second per breath, so if compromised by small increases in mucus production, or thickening, breathing and hence performance may be impaired (Oikawa *et al.* 1994). Although the disease is classified as “poor performance syndrome”, this is a relatively mild form of disease, of which pneumonia and pleuropneumonia are related and much more severe. *A. equuli* and a beta haemolytic Streptococcus have been isolated together from cases of pleuropneumonia with pleural effusion (Sweeney and Maxson, 1995), confirming that lower airway disease involving these particular bacteria may vary in severity. Several uncontrolled field studies confirm these findings, that β haemolytic streptococci (most of which are likely to be *S. zooepidemicus*), *S. pneumoniae* and *Pasteurella* sp. or *Actinobacillus* sp. are commonly isolated from tracheal washes (Sweeney *et al.* 1985, 1989, 1991). Recent evidence suggested that *Mycoplasma felis* and *Mycoplasma equihirinis* were also associated with equine lower airway disease (Wood *et al.* 1994, 1996), but *S. pneumoniae* was associated with inflammation of the upper airway (Wood, 1996). *Mycoplasma* spp. have been isolated from the respiratory tract of both diseased and clinically normal horses before (reviewed by Beech and Sweeney, 1991), but until now, its association with disease has been unknown. Disease was defined as an increase in the amount of mucus visible during endoscopy, an

increased proportion of neutrophils and a nucleated cell count of over 1000 per ml in the tracheal wash (Whitwell and Greet, 1984).

A number of other bacterial species have been associated with the equine respiratory tract. Investigations suggest that LAD is common in Thoroughbred foals as well as young horses in training and is likely to have a bacterial aetiology (Hoffman *et al.* 1993). *Rhodococcus equi* causes pneumonia with a mortality rate of up to 80% (Ellissalde, Renshaw and Walberg, 1980; Ellenberger and Genetzky, 1986), but it is uncommon in the U.K. *Bordetella bronchiseptica* has long been known as a cause of respiratory illness in dogs and swine, and has more recently been recognized in horses with signs of respiratory disease. It was cultured from nasal discharge (Gallagher, 1965; Vandevemme *et al.* 1995), the tracheal washes of horses aged 2 years or less, and older horses which had halothane anaesthesia in the 7 days preceding. Miller (1965) isolated *B. bronchiseptica* from a horse with conjunctivitis and respiratory infection. The guttural pouch was an isolation site for cases of chronic pharyngitis (Noren, 1985) and strangles - like symptoms (Cockram and Webb, 1981). *Klebsiella* spp., *Escherichia coli*, *Staphylococcus* spp., *Pseudomonas* spp. and *Nocardia* spp. can also be isolated from the respiratory tract, but often with no clear association with disease. There are a few species of pathogenic fungi, but they tend not to occur much in the U.K.; *Aspergillus*, *Phycomycetes* and *Candida* spp. only tend to cause problems in the immunocompromised patient. Parasitic infections of the lung are also uncommon in domesticated horses in the U.K. since most are wormed routinely, but *Dictyocaulus arnfieldi*, *Parascaris equorum* and *Pneumocystis carinii* can affect the respiratory tract.

1.3.2.3 Immune mechanisms during bacterial infection

Whether an infectious agent causes disease depends upon the immune system of the host as well as the virulence of the microbe. For instance, macrophages in the lung are a primary defence mechanism, but stress caused by transport or intense exercise has been associated with declining alveolar macrophage function (Laegreid *et al.* 1988).

When bacteria enter the lung, there is an influx of neutrophils which mediate phagocytosis and killing (Newhouse, Sanchis and Bienenstock, 1976). Several common bacterial components activate the alternative complement pathway, with consequent release of C3a and C5a, opsonisation of bacteria and possibly complement mediated lysis; activation of neutrophils and macrophages; triggering of cytokine release; mast cell degranulation leading to increased blood flow in the local capillary network; increased adhesion of cells and fibrin to endothelial cells. The clotting system and fibrin formation may also be activated, limiting bacterial spread. Antigen is processed and presented to helper T lymphocytes, which produce interleukins to activate macrophages, and B lymphocytes to differentiate into antibody producing plasma cells. The lymphoid tissue associated with the bronchus is the main area of antigen presentation and immunoglobulin production in the horse (Warner, 1990).

The immune response to infection can cause tissue damage, which tends to lower pH, particularly as intracellular enzymes are released; for instance, *P. haemolytica* can inflict severe pulmonary tissue damage to calves through the response of neutrophils (Breider *et al.* 1988). *P. haemolytica* can also mediate more subtle changes to the pulmonary vascular system, resulting in oedema (Greenlees *et al.* 1987) and its lipopolysaccharide (LPS) can induce the production of inflammatory cytokines in bovine alveolar macrophages (Yoo *et al.* 1995).

The LPS characteristic of gram negative bacteria can be responsible for some of the clinical and histopathological signs in a disease process, even though some effects may be beneficial to the host in clearing the infection. Interleukin 1 (IL-1) is thought to enhance non-specific host defences; it is synthesised in response to LPS and released by macrophages, where it acts upon the hypothalamus to raise the body temperature, resulting in fever (Kenedi *et al.* 1982), which in turn increases leucocyte mobility and killing of bacteria (Roberts and Steigbigel, 1977; Sebag, Reed and Williams, 1977) and enhances proliferation and transformation of lymphocytes (Ashman and Nahmias, 1977; Roberts and Steigbigel, 1977). Hypoferraemia is also triggered by IL-1, which is the reduction of

plasma iron and zinc, by storage in the liver and absorption by the gut, further starving pathogens of iron.

LPS and lipoteichoic acid (LTA) present in Gram positive bacteria induce the production of interferon gamma, which activates killing mechanisms by macrophages and recruits circulating neutrophils and lymphocytes to the site of infection, and Tumour necrosis factor alpha production by monocytes and macrophages, which activates phagocytic cells. At high levels both of these cytokines can cause considerable damage to the host. LTA can directly induce macrophages to produce IL-12, which induces the T cell responses and further increases interferon gamma production. IL-12, TNF alpha and IL-1 act synergistically to induce proliferation of Th1 clones (Trinchieri and Scott, 1994). Although there is information available about the interactions between bacteria and the host generally, little specific information is available on interactions between the equine host and bacterial pathogens.

1.3.2.4 Treatment and prevention of infection/transmission

Nursing care which promotes the patient's appetite and good attitude, is an important contribution to a favourable outcome (Beech and Sweeney, 1991). Adequate ventilation with minimal dust should be provided for healthy horses as a preventative measure, as well as for diseased animals, since inadequate ventilation, when combined with other pollutants such as ammonia and dust, damages the nasal and epithelial tissue (Kovacs *et al.* 1967; Martin, 1970; Martin and Willoughby, 1972; Doig and Willoughby, 1971; Jones *et al.* 1987). Mucking out should not take place with the horse in the stable where possible, to minimize exposure to airborne particles, which increase at such times (Favell, 1996). A controlled study found a higher incidence of lower respiratory tract disease in horses kept in loose boxes on straw bedding, than those housed in a barn on paper bedding (Burrell *et al.* 1982). Patients should be rested and not transported or otherwise stressed (Beech and Sweeney, 1991), since transport was associated with respiratory compromise and disease (Oikawa *et al.* 1994; Raidal, Love and Bailey, 1996). Also, food and water receptacles should be placed on the floor, since keeping the head up slows mucociliary transport

(Raidal, Love and Bailey, 1996). Isolation of affected animals and attention to hygiene, such as disinfecting grooming tools and rugs, are suggested to be useful in restricting the spread of disease, since personnel or equipment can transfer bacteria from horse to horse.

Treatment with antibiotics is the usual course of action in these cases, because if 10^4 or more bacterial colony forming units (cfu) per ml are cultured from a tracheal wash, they are likely to be damaging the horse's lungs and / or trachea, whether they are primary or secondary pathogens (Wood and Chanter, 1994). Antibiotics are chosen, based on the *in vitro* sensitivity of the bacteria (Wood and Chanter, 1994). Penicillin can be rendered ineffective by acid pH and the presence of pus, and some *Pasteurella* isolates and *A. pleuropneumoniae* produce a constitutive penicillinase in any case (Girard *et al.* 1987), but erythromycin however, penetrates the lungs, pleural fluid and neutrophils. Other drugs which may be effective include chloramphenicol, cephalosporins, trimethoprim-sulphonamide combinations and synthetic penicillins. Immune modulating drugs are of unknown efficacy in the treatment of bacterial LAD of the horse. Non-steroidal anti-inflammatory drugs may help to alleviate symptoms, but they are antipyretic, which may hinder temperature monitoring as a guide to therapeutic efficacy of antibiotics (Beech and Sweeney, 1991). Use of sulphanilamide has been reported as effective for the treatment of pneumonic horses bacteraemic with haemolytic streptococci (Boddie, 1939). At present, there are no effective vaccines for bacterial LAD since until relatively recently, they were mostly considered opportunistic colonizers, secondary to viruses, but the demand for a vaccine is likely to increase as knowledge of the etiology and prevalence is disseminated among practitioners.

1.3.3 *Streptococcus zooepidemicus* and related bacterial pathogens

1.3.3.1 Classification

The first classification of the genus *Streptococcus* by Brown (1919), was based on haemolytic reactions seen on blood agar plates, which are alpha, beta and gamma; *S. zooepidemicus* is beta haemolytic, which produces a clear zone of haemolysis on blood agar. Rebecca Lancefield (1933) further differentiated the beta haemolytic organisms by

group specific carbohydrate antigens named A-O. In 1937, Sherman divided the genus into four groups: pyogenic, viridans, lactic and enterococci. *S. zooepidemicus* is part of the pyogenic group, which also includes *S. equi* and *S. pyogenes*. Comparative 16S rRNA phylogenetic trees constructed by Bentley, Leigh and Collins (1991), suggested that *S. equi* was closely related to *S. pyogenes*. Kilper Bältz and Schleifer (1984) reviewed comparative 16S data, hybridization studies and cell wall analysis to make a phylogenetic tree which was in agreement with *Bergey's Manual of Determinative Bacteriology*, which recognizes *S. equi*, *S. zooepidemicus* and *S. equisimilis* as subspecies of *S. equi*, but Chanter *et al.* (1996), suggested that *S. equi* was more likely to be a subspecies of *S. zooepidemicus*.

1.3.3.2 *S. zooepidemicus* disease in mammals

S. zooepidemicus is present in the normal flora of equines. It has been isolated from the mouth, nose, respiratory and genital tracts of healthy individuals (Welsh, 1984). Woolcock (1975) isolated mainly *S. zooepidemicus* from tonsils, throat, mandibular and pharyngeal lymph nodes. Kester, Lesser and Dowd (1993) found that *S. zooepidemicus* was isolated from 34% of cases of equine respiratory disease. Nevertheless, it can be pathogenic in certain circumstances to equines, other domestic animals and occasionally man. *S. zooepidemicus* and *S. equi* have only been isolated from patients with what were believed to be zoonotic infections (Cimolai *et al.* 1988; Ghoneim & Cooke, 1980), particularly following ingestion of unpasteurised milk (Barnham *et al.* 1983; Duca *et al.* 1969; Edwards *et al.* 1988), or contact with pigs (Yuen *et al.* 1990).

S. zooepidemicus can significantly affect fertility. It causes placentitis, endometritis (Causey, Todd & Paccamonti, 1994), abortion and stillbirth in the mare (Hong *et al.* 1993). Stallions can succumb following infections carried in semen, which causes a reduction in the numbers and quality of spermatozoa. It is also associated with inflammatory infection, particularly wounds such as bites and burns. It has also been isolated from deep seated abscesses in such sites as the brain, lung and abdominal tissue. It has been associated with septicaemia, infectious arthritis of joints and occasionally the digestive tract.

1.3.3.3 Pathogenic determinants

Streptococci produce a number of virulence factors, including membrane damaging streptolysins O and S, pyogenic exotoxins (superantigens), hyaluronidase, streptokinase, proteases, (particularly C5a peptidase, which cleaves human serum chemotaxin C5a at the polymorph binding site, fibronectin binding proteins (adhesins) and IgG/IgA binding proteins.

Much of the work done on pathogenic streptococci has been carried out with *S. pyogenes*, which causes glomerulonephritis, rheumatic fever (Fischetti, 1989) and toxic shock. M proteins are thought to be major protective antigens (Lancefield, 1962, Fischetti, 1989, Kehoe, 1991), which are members of a larger family of M-like proteins, which probably arose through gene duplication and divergence (Fischetti, 1989), which include the group C and G streptococci. There are over 100 types of *S. pyogenes* based on amino terminal variability (Kehoe, 1991). They have sequence homology with mammalian proteins (Fischetti, 1989), which can induce a harmful host response due to cross-reactive antibody (Froude *et al*, 1989). They are also antiphagocytic (Kehoe, 1991). They inhibit non-immune opsonisation by binding fibrinogen and complement factor H. It is suggested that plasma fibrinogen masks C3b binding sites on the cell surface and binding of factor H inhibits alternative pathway C3 convertase and classical C5 convertase (Hong *et al*, 1990). Subsequent phagocytosis by C3b receptor bearing neutrophils would be prevented. Immune opsonisation is not inhibited by fibrinogen or factor H. Antibody directed to the extreme amino terminal end of the M protein opsonises still in the presence of bound fibrinogen (Ryc *et al*, 1989). Immunity in humans and experimental animals is due to type specific opsonic antibody to the variable amino terminal half of the M protein.

Proteins in hot acid extracts elicit the production of opsonic antibody in both *S. zonepidemicus* and the related *S. equi*, the causative agent of strangles in equids. However, whilst *S. equi* M-like protein is antigenically conserved, M-like protein of *S. zonepidemicus* is heterogeneous in antigenicity and molecular weight. Despite the similarity between the two, shown by DNA hybridisation studies, they each have distinct

pathogenicities and *S. zooepidemicus* has a broader range of host species (Farrow and Collins, 1984).

Type specific antibody to M-like protein may play an important role in susceptibility and recovery in the horse. *S. zooepidemicus* may be similar to *Streptococcus pyogenes* with respect to cross- protection between strains with different M-like proteins. *S. pyogenes* has many serotypes of M protein; usually a single serotype is expressed by each strain. Immunity in man is conferred by a type-specific opsonic anti M protein antibody, with no reported cross-protection between types. Moore and Bryans, (1970), described at least 15 serotypes of M-like proteins in *S. zooepidemicus* isolates from Europe and the USA on the basis of immunoprecipitation using cross-absorbed antisera.

Some bacteria possess capsules made of hyaluronic acid (HA), or hyaluronate, which is a polymer of high molecular mass, comprising equimolar proportions of D-glucuronic acid and N-acetyl-D-glucosamine and is of similar composition, regardless of the source (Meyer *et al.* 1956). HA from Lancefield Group A and C Streptococci is chemically indistinguishable from mammalian hyaluronate (Froude *et al.* 1989). Streptococci of groups A and C, *S. uberis* and *Pasteurella multocida* type A (Pandit and Smith, 1993), are the only well characterized prokaryotes that produce a HA capsule, although other species produce capsular polysaccharide. HA is common throughout the animal kingdom in vertebrates and invertebrates. It is one of the main components of the extracellular matrix in mammals, which influences cell-cell adhesion (Pessac and Defendi, 1972; Underhill and Dorfman, 1978), stimulates locomotion and phagocytosis (Hakansson, Hallgren and Venge, 1980; Hakansson and Venge, 1985), and modulates angiogenesis (Feinberg and Beebe, 1983; West *et al.* 1985). It is concentrated in parts of the body, where it has a supporting and cushioning function, in the joints and the anterior segment of the eye (Rittig *et al.* 1993).

The locus encoding capsule characterized for group A Streptococci encodes hyaluronate synthase (*HasA*) (DeAngelis, Papaconstantinou and Weigel, 1993; Dougherty and van de

Rijn, 1994), UDP-glucose dehydrogenase (*HasB*) (Dougherty and van de Rijn, 1993) and a gene with homology to UDP-glucose pyrophosphorylase (*HasC*) (Crater and van de Rijn, 1995). Hyaluronate capsule is thought to be a virulence factor for *S. pyogenes* (Kass and Seastone, 1944; Wessels *et al.* 1991) and group C Streptococci (Hirst, 1941). *S. pyogenes* acapsular mutants, which had no functional hyaluronate synthase enzyme, were avirulent in mice that suggested that the capsule may prevent phagocytosis (Wessels *et al.* 1994). Further investigation suggested that capsule was variably important in the mechanism of resistance to opsonisation and phagocytic killing (Dale *et al.* 1996; Moses *et al.* 1997). Ability to resist phagocytosis in the non-immune host correlated with virulence, and this might be due at least in part to the ability of HA to be degraded by free radicals (Schenck *et al.* 1995), hence scavenging free radicals produced by the host and protecting membranes. Alternatively, host phagocytic cells may be repelled by a net negative charge (Whitnack, Bisno and Beachey, 1981). Possession of capsule for group B Streptococci, although not made of HA, was also correlated with virulence (Rubens *et al.* 1987). Chanter *et al.*, 1994, showed that the increased resistance of young cultures of *S. equi* to phagocytosis by polymorphonuclear leukocytes was due in part to a much elevated rate of capsule production, whilst Griffith, (1923) demonstrated that mixing heat killed virulent encapsulated *S. pneumoniae*, with non-encapsulated avirulent cells, yielded encapsulated bacteria which were lethal for mice.

Hyaluronate synthase (HS) is an enzyme thought to have a universally similar mechanism, with the exception of plants (Feingold and Franzen, 1981). HS is thought to reside in the plasma membranes of eukaryotic cells, where it synthesizes HA into the extracellular matrix (Prehm, 1984); HS resides in the protoplast membrane of Streptococci (Markovitz and Dorfman, 1962). The bovine enzyme has been studied in the most detail and was found to be a UDP-glucose dehydrogenase, made of three dimeric 52 KDa subunits, which catalyses the formation of UDP-glucuronic acid. The equivalent enzyme in *S. pyogenes* was cloned and the 47 KDa protein expressed was demonstrated to have HS activity (DeAngelis, Papaconstantinou and Weigel, 1993). The DNA sequence was found to be similar to the

Pseudomonas aeruginosa AlgD sequence, a dehydrogenase which catalyses GDP-mannuronic acid production for the alginate capsule (Dougherty and van de Rijn, 1993).

A protein which bound precursor nucleotide sugars and was complexed with nascent hyaluronate, so was originally designated as HS, was isolated from *Streptococcus equisimilis* cholate-solubilized protoplast membranes (Prehm and Mausolf, 1986) and was later cloned and sequenced, and found to be 56KDa (Lansing *et al.* 1993). Antibody to the 56 KDa protein cross-reacted with eukaryotic plasma membrane protein (Mausolf *et al.* 1990). At the same time, Triscott and van de Rijn (1986) found that a 42 KDa HS could be isolated from *S. equisimilis* digitonin-solubilized membranes. This inconsistency between the 56 and 42 KDa proteins was not fully explained until DeAngelis, Yang and Weigel, (1994), used a sequence database to assess the similarity of the *S. pyogenes* HS to other gene products. They found that the 42 KDa protein from *S. equisimilis* was very similar, although not identical to the sequence deduced for *S. pyogenes*, but different to the 56 KDa protein previously described by Prehm and Mausolf, (1986), and Lansing *et al.* (1993). Subsequent work has purified a 42 KDa HS from *S. equisimilis* (Prehm, Nickel and Prehm, in Press).

The 56 KDa protein is now thought to be a threonine kinase, but more work is required to confirm this (Prehm, personal communication, 1996). Antisera to the *S. equisimilis* 56 KDa protein reacted with a 50 KDa eukaryotic protein, (Prehm 1989), which was known to catalyse the alternate addition of the substrate sugars UDP-glucuronic acid and UDP-*N*-acetylglucosamine (Prehm, 1983), a different reaction to that described as being catalyzed by HS. This eukaryotic enzyme required phosphorylation to attain its active form, and was important for fibroblast detachment during mitosis (Prehm, 1989). Less is known about the *S. equisimilis* enzyme, although work carried out when it was thought to be the HS, demonstrated that it is shed associated with hyaluronate into the medium of growing cultures (Mausolf *et al.* 1990), rather than residing in the protoplast membrane like Streptococcal HS (Markovitz and Dorfman, 1962).

1.3.3.4 Vaccination strategies

Although epidemiological data indicated an association between *S. zooepidemicus* and equine respiratory disease, Koch's postulates have not been fulfilled, which is necessary if a candidate vaccine is to be tested. An equine experimental model could be used to test for the fulfillment of Koch's postulates in this instance and provide a way of testing *in vivo* the efficacy of trial vaccines. A septicæmic model of *S. zooepidemicus* disease in the equine exists, (Varma *et al*, 1984), but a respiratory challenge model would be more realistic for an investigation of lower airway disease.

There are no reports in the literature of *S. zooepidemicus* vaccination, although prevention of the related *S. equi* has a long history, which was reviewed by Todd (1910). The Russians, who exposed their horses to extreme cold in an attempt to reduce strangles mortality. The first vaccination was reported by Jensen and Sand, who injected cultures intravenously; this was said to protect against challenge, but had dangerous side effects. Kitt and Gabritchewski (1906) protected foals from nasal challenge with killed cultures, but efforts in the early twentieth century were mostly directed towards the use of convalescent serum to protect against strangles and to reduce the symptoms of affected horses (Delvos, Rohrs and Jacoulet). Danonville and Wissocq (1905) produced therapeutic hyperimmune sera, but this was expensive and found to be short lived (Todd, 1910).

Bazely (1942) found that heat inactivated young cultures of *S. equi* protected mice, so in the first fully documented field test, vaccinated 2486 horses with a similar preparation and compared the incidence of strangles with another group of 1922 horses. 1.17% of the vaccinates developed strangles, compared with 5.26% of the controls, which was considered significant, but the work was ignored for years. Twenty-two years later, Bryans, Doll and Shephard (1964), conducted a similar trial of a similar vaccine, and again found that disease incidence was reduced following vaccination, so this time, the preparation was sold commercially.

Only five years later, Engelbrecht (1969) published complaints that vaccination gave rise to local reactions and breaks in immunity, but these were considered infrequent and it was said that the vaccine was generally accepted. One of the problems with strangles, is that some animals can recover from infection and become asymptomatic carriers of *S. equi*, who have type specific immunity, but still can infect healthy susceptible animals (Woolcock, 1975). Efficacy of vaccination was later questioned by Srivastava and Barnum (1981), who found that outbreaks of strangles had been occurring annually in Canada since 1975, despite a vaccination programme. They tried to improve the vaccine formulation by giving ponies mixtures of formalin treated cells and hot acid extracts; ponies were still only partially protected from challenge, so they tried M protein alone, mixed with aluminium hydroxide adjuvant, but again only achieved partial protection (Srivastava and Barnum, 1983). At the same time, another paper questioned the efficacy of bacterin based strangles vaccines (Anon, 1983).

Srivastava and Barnum (1985) then investigated why protection was incomplete, and suggested that the immune response following vaccination with several doses of bacterin was short-lived. At the same time, Timoney and Eggers (1985) suggested that the role of serum bactericidal antibody was overrated, and found that animals convalescing from challenge were resistant to ten times the dose of *S. equi* and had antibody to M protein in the nasopharyngeal mucus (Galan and Timoney, 1985).

Now it was more widely acknowledged that vaccination was not fully protective and could give rise to adverse reactions, efforts concentrated on identifying which animals might derive the most benefit from vaccination, formulating products with fewer side effects and identifying what factors reduced the efficacy of vaccination programmes. A study of foals inoculated with three doses of M protein based vaccine, found that they were less than half as likely to have clinical signs of strangles as non-vaccinates (Hoffman *et al.* 1991). The implication was that it was worth vaccinating a naive foal if it was to be in contact with strangles rather than risking complications, but there were side effects and questions over long term protection that meant that the risks might outweigh the benefits. A survey of

vaccination in the field found that the recommended program was rarely followed (Jorm, 1990), although this survey was criticised by Fairley (1991), highlighting the difficulties of field evaluation and giving possible reasons why problems with vaccination took so long to emerge. To illustrate this point, Bryant *et al.*, (1985), found that the efficacy of mutanolysin extracts of *S. equi* depended upon the severity of the challenge.

Vaccines based upon cell free M protein extracts largely replaced bacterins, because they had fewer side effects (Smith, 1994), although Timoney (1993), found that commercially available hot acid extracts, or mutanolysin extracted M protein, occasionally caused purpura haemorrhagica. This is a serious allergic reaction to antigen, where antigen-antibody complexes circulate and damage the blood vessel linings, allowing blood to escape into the tissues; even with careful nursing, only 50% of horses recover.

Newer approaches to strangles vaccination have involved recombinant DNA technology. Galan and Timoney (1987), constructed a genetically modified avirulent *E. coli*, which expressed M protein antigens of *S. equi* and a stable, avirulent bacteriophage modified *S. equi* has also been produced (Timoney, 1993). Wallace *et al.* (1995) tried formalin killed *S. equi* orally and intraperitoneally, live avirulent *S. equi* and microencapsulated M protein with a rat model of strangles, and from this, formalin killed and live avirulent *S. equi* were chosen for study in horses. These only resulted in partial protection to horses, meaning that vaccinates were less ill than controls.

At present, there is still no success in finding a vaccine against strangles that is safe, fully protective and halts the spread of disease. Studies with monoclonal antibody to M protein suggest that it is protective in mice (Jean-Francois *et al.* 1991), but serum antibody alone is not fully protective in the horse, so it is likely that some other mechanism might be helping *S. equi* to evade the immune response, because phagocytic cells migrate to the site of a lesion, but do not ingest the bacteria. Young cultures of *S. equi* can be phagocytosed in the presence of specific antibody if the thick hyaluronate capsule is removed (Chanter *et al.*

1994). This suggests that one of the ways forward for strangles research, like research into *S. pyogenes*, is to investigate ways of dealing with both capsule and M protein.

For *S. zooepidemicus* vaccination, such an approach might be equally valid, although unlike *S. equi*, *S. zooepidemicus* is thought to have a number of different M-types, rather like *S. pyogenes*.

1.3.4 Actinobacillus equuli and related pathogens

1.3.4.1 Classification

The classification of *Actinobacillus equuli* has varied, which is reflected in a number of name changes in the past (reviewed by Kim, 1976). *A. equuli*, and *A. suis* from the oral cavity of horses were provisionally designated as Taxon 11 by Bisgaard *et al.*, (1984), as they are distinct from porcine *A. suis*, but Bergey's Manual of Determinative Bacteriology (Holt, 1994), has not been amended. *A. equuli* is a pleomorphic gram-negative bacterium, currently classified as part of the family Pasteurellaceae, which includes the genera, *Actinobacillus*, *Pasteurella* and *Haemophilus* (Holt *et al.* 1994). There were found to be antigenic relationships between *Pasteurella* and *Actinobacillus* species (Mraz, 1977).

More recently, molecular techniques have been employed to determine relatedness between members of the Pasteurellaceae. An early DNA-DNA hybridisation study suggested that *A. equuli* was closely related to *A. suis*, *P. ureae*, *A. ligniersii* and *A. capsulatus*, but not to *P. haemolytica* (Escande *et al.* 1984), but Mutters *et al.* (1986), used DNA-DNA or DNA-rRNA data, to confirm that *P. haemolytica* should be grouped with the Actinobacillaceae. Borr *et al.* (1991), used both DNA hybridization and restriction endonuclease fingerprinting, and concluded that *A. equuli*, *A. suis* and *A. ligniersii* were close relatives. *A. pleuropneumoniae* was also found to be closely related, whilst *P. haemolytica* was found to share some homology. Dewhirst *et al.* (1992) used 16S ribosomal RNA studies and agreed with the conclusion of Borr *et al.*, but along with *P. haemolytica*, also grouped *H. parahaemolyticus*, *H. parainfluenzae*, *H. paraphrohaemolyticus*, *H. ducreyi*, Bisgaard Taxon 11 and *A. ureae* in together with *A. equuli* and the others.

Confusion about the placing of *P. haemolytica* might be due to the fact that two biotypes, each with distinct pathogenicities have been identified, A and T, based upon fermentation of arabinose and trehalose respectively. Classification into separate species was proposed (Tsai, Collins and Hoiby, 1988; Murray *et al.*, 1992) and *P. haemolytica* now refers to the A biotype, whilst the T biotype is now known as *P. trehalosi* (Holt *et al.* 1994).

1.3.4.2 *A. equuli* and related disease in mammals

In equines, *A. equuli* is widely recognized as a cause of septicaemic disease leading to neonatal death in foals (Bain 1954, Platt 1973). Causes of death include pneumonia, abscess, arthritis and peritonitis, for which there are a number of case reports around the world (Lutzelschwab *et al.*, 1987; Carter and Martens, 1986; Lee *et al.*, 1985). One analysis of foal losses found that bacterial septicaemia, some caused by *A. equuli*, pneumonia and arthritis were significant causes of loss. Particularly affected are foals which were colostrum deficient, or there was failure of colostrum immunoglobulin transfer (Vaissaire *et al.* 1988; Kamada *et al.*, 1985). Studies of the causes of perinatal mortality found that bacteria, including *A. equuli*, were isolated from many foals, whilst virological examinations, where performed were usually negative (Joseph and Devendran, 1987; Vaissaire *et al.*, 1987; Kanemaru *et al.*, 1985).

Isolates from older horses have previously been regarded as commensal and unlikely to be pathogenic (Smith 1965); haemolytic *A. equuli* and *A. suis*-like bacteria are thought to be part of the normal flora of the oral cavity (Bisgaard *et al.*, 1984). *A. equuli* was however, isolated from cases of pericarditis (Dill *et al.* 1982), retropharyngeal infection (Todhunter, Brown and Stickle, 1985), abscess (Vaissaire *et al.* 1988), peritonitis (Gay and Lording, 1980) and infection of the gastrointestinal mucosa (Al-Mashat and Taylor, 1986). Abortion in mares can be the result of *A. equuli* infection (Webb, Cockram and Pryde, 1976) and it is sometimes present in the milk (Vaissaire *et al.* 1988); *A. equuli* was isolated from routine cervical swabs, but was uncommon in the Hannover breed tested (Fluge, Kruger and Scherbarth, 1972). In stallions, *A. equuli* products are thought to affect fertility, as *in vitro*

data suggested that culture filtrate reduced sperm motility; *A. equuli* was isolated from a case of periorchitis (Belknap, Arden and Yamini, 1988).

A. equuli was also associated with mortality in the Cape mountain zebra (Penzhorn, 1984), which is of the genus *Equus*, a close relative of the horse. Amongst other mammals, *A. equuli* is found in diseased swine (Schwartz, 1986, Windsor, 1973), and has been isolated from adults and piglets with septicaemia or joint inflammation, endocarditis, osteomyelitis and embolic pneumonia (Pedersen, 1977). It has been suggested as a cause of porcine abortion (Werdin *et al.* 1976) and was isolated from dead neonatal piglets, whom were thought to be infected from the sow, who was in turn thought to be infected from horses present at the market (Okolo, 1987).

Related bacterial species are principally respiratory pathogens. *A. suis* and *A. pleuropneumoniae* cause severe pleuropneumonia in pigs (Sebunya and Saunders, 1983), whilst *P. haemolytica* causes pneumonia in sheep and lambs (Malone *et al.* 1988), goats (Zamri *et al.* 1987), cattle (Devreise *et al.* 1987; Fischer *et al.* 1987) and fowls (Infante, *et al.* 1984; Lin, 1986).

1.3.4.3 Pathogenic determinants

Knowledge of the pathogenic mechanisms of *A. equuli* and related bacteria in the horse is scant, but most pathogenic bacteria, including some which are related to *A. equuli*, have mechanisms which enable them to survive and multiply in the sometimes hostile environment provided by the host.

Firstly, bacterial respiratory pathogens need a mechanism which enables colonisation of the respiratory epithelium and avoids removal by the host mucociliary escalator; *P. haemolytica* adheres to calf respiratory epithelia *in vitro*, in a manner dependent on the number of bacteria and contact time (Muller *et al.* 1988).

Toxins are important virulence determinants in a number of pathogenic bacteria, including *A. pleuropneumoniae*. Three exotoxins, APX I, II and III all belonging to the pore-forming RTX toxins, have been discovered amongst the serotypes (Frey *et al.* 1993). Each serotype has been found to secrete one, or a combination of two of the toxins. The degree of virulence of the serotypes is associated with the toxins expressed (Beck *et al.* 1994). All form cation selective channels of different sizes. Expression of APX I is associated with severe outbreaks of pleuropneumonia; it is haemolytic for swine red blood cells and cytotoxic, producing the most channels in host cell membranes. APX II is weakly haemolytic and the least cytotoxic, producing the fewest channels. *P. haemolytica* also produces a leucotoxin (Chang *et al.* 1987), which is similar to APX II. APX III is non-haemolytic, but strongly cytotoxic, producing the second most channels.

There is a large amount of iron in vertebrate body fluids, but only 10^{-18}M is potentially available for bacterial growth (Bullen *et al.*, 1978, Chrichton and Charleaux-Wauters, 1987, Weinberg, 1990). Fe(III) has a low solubility *in vivo* and is mostly associated with carrier proteins such as haemoglobin within red blood cells, ferritin and myoglobin (Lanskowsky, 1976). Transferrin, which supplies iron to the bone marrow for haemoglobin synthesis, is present mainly in the blood (Aisen and Leibman, 1972), whilst lactoferrin occurs intracellularly and in secretions. Both maintain iron stores as a small proportion of the total iron and have a high iron association constant, so little is free for bacterial utilization (Bullen *et al.*, 1978). In cases of severe inflammation, the concentration of serum iron declines (hypoferraemia), because iron free lactoferrin released from neutrophils removes iron from holotransferrin; iron-lactoferrin complexes are then taken up by macrophages, which have membrane bound lactoferrin receptors and are rapidly removed from the circulation (van Snick *et al.*, 1974). Increased ferritin synthesis in the liver, which diverts labile iron into ferritin stores, further reduces the concentration of extracellular available iron in tissue (Konijn and Hershko, 1977).

Some bacteria possess methods of specifically acquiring iron which promote or are required

for survival and multiplication in the host. There are two main iron uptake systems (reviewed by Otto, Verweij-van Vught and Maclaren, 1992): These are i) Synthesis of low molecular weight siderophores which sequester iron from transferrin and lactoferrin, then convey it to the bacterium where interaction with a specific outer membrane receptor internalises the iron; examples include *Escherichia*, *Salmonella*, *Klebsiella*, *Shiga* and *Enterobacter* species. ii) Direct interaction with transferrin, lactoferrin, or haem containing proteins via a specific bacterial outer membrane receptor; examples include, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, which can use transferrin, lactoferrin and haem-compounds for growth. Outer membrane proteins produced by bacteria only in conditions where the amount of available iron is limited are known variously as iron regulated proteins (IRPs), or iron regulated outer membrane proteins (IROMPs).

The epidemiology and pathogenic mechanisms of *A. equuli* and related bacteria in the horse are not well characterized. In particular, there is no record of outer membrane protein antigen characterization from equine tracheal isolates, nor studies of their relatedness. Kim (1976), divided 128 foal *A. equuli* isolates into 28 serogroups using an immunodiffusion precipitin test that differentiated the heat stable O antigens, but no serogroups were consistently associated with disease. Serotyping based on proteins produced in response to iron limitation has not been previously investigated. It is not known whether *A. equuli* produce IRPs which could elicit the production of antibody, or how isolates are antigenically related.

A. pleuropneumoniae and *P. haemolytica* are thought to bind holotransferrin directly to the cell surface via a receptor in iron limited conditions; IRPs are produced by both. Another potential virulence determinant, described for *A. pleuropneumoniae* is the production of a secreted copper/ zinc superoxide dismutase (SodA). It is speculated that it accelerates the dismutation of superoxide derived from neutrophils. Interestingly, the production of sodA by *A. pleuropneumoniae* increases anaerobically only with iron restriction (Langford, Loynds and Kroll, 1996).

1.3.4.4 Vaccination strategies

Although epidemiological data indicated an association between *Actinobacillus/Pasteurella* species and equine respiratory disease, Koch's postulates have not been fulfilled, which is necessary if a candidate vaccine is to be tested. An equine experimental model could be used to test for the fulfilment of Koch's postulates in this instance and provide a way of testing *in vivo* the efficacy of trial vaccines. There are no animal models documented for *A. equuli*, although challenge models in the taxonomically related *P. haemolytica* and *A. pleuropneumoniae* have been successfully developed for cattle and sheep, and pigs, respectively. IRPs are produced by *P. haemolytica* are immunogenic and sodium salicylate extracts of iron limited cultures containing IRPs, successfully protect specific pathogen free lambs against the development of pneumonic lesions (Gilmour *et al*, 1991). The intranasal immunization route was demonstrated to protect calves against pasteurellosis (Klein, 1987).

For a vaccine, it is also essential to know whether cross-reactive bacterial components are present, to gain an impression of how many strains to put into a vaccine. Vaccines to protect pigs against *A. pleuropneumoniae* infection originally consisted of killed bacteria mixed with an adjuvant; these gave protection against homologous, but not heterologous serotypes (Nielsen, 1988). Similarly, experimental infection protected against homologous challenge, but the degree of protection against heterologous challenge varied (Crujisen *et al*. 1995i). One author concluded that vaccination should be combined with the use of antibiotics, whilst another found that isolating affected animals was as effective as medication and vaccination in controlling an outbreak (Clark *et al*. 1994). The environment the pigs were kept in was also judged to have a role in the pathogenesis of disease (Beskow, Soderlind and Thafvelin, 1989). Bacterin based vaccines were thought to fail because of antigenic differences in lipopolysaccharide within serotypes (Jolie, Mulks and Thacker, 1995). This indicated that vaccination was not optimally effective and environmental changes were not always practical, so some workers attempted to enhance non-specific responses by the addition of interleukin 2 (Anderson, Fedorka-Cray and Urban, 1988), or *Propionibacterium avidum* (Markowska *et al*. 1992). Studies were also set up to test the efficacy of different adjuvants; oil based ones gave a good response, but the most

extensive tissue reactions (Willson, Rossi-Campos and Potter, 1995). The core components of gram negative bacterial cell walls could prevent mortality, but not infection or disease (Fenwick *et al.* 1986), so the search for vaccine components became more specific to *A. pleuropneumoniae*.

It was suggested that neutralizing antibody to toxins may be important for the induction of protective immunity in pigs (Bertram, 1988; Devenish, Rosendal and Bossé, 1990; Fedorka-Cray *et al.* 1990; Cruijisen *et al.* 1995ii). It was claimed that a haemolysin, when mixed with outer membrane protein extract, gave complete heterologous protection against experimental challenge (Bosch *et al.* 1990). Suggestions have been made that there are other mechanisms of protection besides antibodies to toxins (Haesebrouk *et al.* 1996), such as iron regulated proteins, which could also contribute to protection (Gerlach *et al.* 1993), although most recent evidence still indicates that anti-haemolysin antibodies are important (Furesz *et al.* 1997).

Recent efforts have been made to design a live, attenuated vaccine. A genetically defined riboflavin auxotroph which does not give rise to signs of disease at 500 times the LD50 dose has been described, but it has yet to be tested in a porcine challenge (Fuller, Thacker and Mulks, 1996), and it remains to be seen whether attenuated strains will protect against challenge by heterologous strains. Taken together, the evidence so far suggests that the control of *A. pleuropneumoniae* in swine would be likely to involve a vaccine with a number of components, combined with improvements to the animals' environment.

The history of *P. haemolytica* vaccination in sheep is similar to that for *A. pleuropneumoniae*. Experimental challenge models were developed for pneumonic Pasteurellosis. Initially, bacterin based vaccines were tried experimentally and sold commercially, but their success was limited to protection from homologous challenge because serotypes were poorly cross-reactive. Sodium salicylate extracts, which contained the proteins in the outer bacterial cell membrane were more successful, particularly when bacteria were cultured in iron limited conditions (Gilmour *et al.* 1991).

1.3.4. Justification for study

It is thought that bacteria, whether primary or secondary agents, possibly cause more damage to the equine lower respiratory tract than any other group of pathogens, so it is probably most beneficial to vaccinate against those most strongly associated with equine lower airway disease (Wood and Chanter, 1994). *S. zooepidemicus* and *A. equuli* were chosen for this study because they were most strongly associated with equine lower airway disease and most frequently isolated (Wood, 1996). As both of these bacteria can be present in the equine without producing disease, there must be a fine balance in the battle of survival between microbe and host. The balance may be affected by the size of the infecting dose and causes which diminish host resistance, such as stress (Rossdale, 1995). Conversely, the balance may be influenced to favour the host by vaccination. It is helpful that precedents are already set for vaccination research in related bacteria.

This is most commonly a sub-clinical infection, meaning that the airway is inflamed and damaged by the time symptoms are noticed and therapy initiated, causing lost training days for convalescence, spreading infection to other individuals and suffering to the horse which is exercised whilst sick. Antibiotics are commonly used to treat bacterial infections, but may penetrate fibrinous lung tissue poorly; some antibiotics have reduced activity caused by enzymes, purulent exudate, and the acidic environment of infected lung tissue (Aronson and Brownie, 1978). As bacterial respiratory illness is common in the equine, increasingly widespread use of the antibiotics not banned by the Jockey Club, could select resistant pathogens in the longer term, further reducing the number of treatment options for the control of outbreaks. Evidence is available for antibiotic resistance carried on plasmids in *P. haemolytica* and antibiotic treatment failure for calves with pneumonic pasteurellosis has been documented, where it was suggested that methods for assessing antibiotic sensitivity may be inadequate and hence underestimating the problem. Another complication is that infections are often mixed and there are sometimes different patterns of antibiotic resistance between species, necessitating combination therapy, which is expensive and restricts treatment options.

There is an urgent need to discover precisely which *Actinobacillus* /*Pasteurella* species are associated with lower airway inflammation, whether they produce iron regulated proteins upon which a vaccine could be based, whether they bind any host iron carrying compounds, and whether it is possible to produce a model of disease for both bacteria. For *S. zooepidemicus*, it is important to know whether there is any prospect for cross-protection between strains with different M-types, and if the prospects are poor, whether a novel approach using hyaluronate associated proteins as surrogate antigens would induce protective immunity. This thesis will attempt to answer these questions and hence give an indication of whether vaccination against bacterial LAD in the horse is an option worth pursuing.

1.4 AIMS AND OBJECTIVES

The principal requirements of identifying a strategy towards protective immunization against lower airway disease (LAD) in the horse are (a) to identify the variety of pathogens and their virulence determinants upon which vaccination might depend and (b) to provide a model of disease in which to test potential protective immunogens. It is with these overall aims that the following objectives were identified as the subject of this thesis with particular reference to iron scavenging mechanisms of the *Actinobacillus/Pasteurella* group of bacteria, the M-like proteins of *S. zooepidemicus* and the capsule of Lancefield group C Streptococci.

1. To speciate isolates of the *Actinobacillus/Pasteurella* group from cases of lower airway disease and controls to assess if the epidemiological association of this general group of bacteria with the disease can be attributed to a predominant species or to a defined group of species.
2. To investigate the production of iron regulated proteins (IRPs) by the dominant species of the *Actinobacillus/Pasteurella* group associated with LAD with respect (a) to binding of host iron carrier proteins; (b) to the association of IRPs and host iron carrier binding with requirement of host iron carrier protein for growth in iron limited conditions; and (c) to an assessment of the antigenic variability and cross-reactivity of IRPs.
3. To assess the antigenic and opsonogenic variability in M-like protein extracts of temporally and geographically disparate isolates of *S. zooepidemicus* from cases of LAD.
4. To clone and express fragments of a hyaluronate associated protein of *S. equi* and assess their potential to cross-protect against *S. zooepidemicus* challenge of mice and hence as an immunogen with the potential to cross-protect between different types of *S. zooepidemicus*.
5. To assess the feasibility of experimental *S. zooepidemicus* and *A. equuli* challenge in conventional ponies.

2.0 CHAPTER TWO

ACTINOBACILLUS AND PASTEURELLA ISOLATES FROM THE
DISTAL TRACHEA OF HORSES WITH BACTERIAL LOWER AIRWAY
DISEASE.

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2.1.1 List of Tables

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Objectives

A strong association was found between lower airway inflammation and the presence of *Pasteurella* / *Actinobacillus*-like bacteria in the tracheal washes of young Thoroughbred horses in training (Wood *et al.* 1993), but the species involved were not established.

The inflammation scores were collected for each tracheal wash sample to establish if one principal species accounted for the association between lower airway inflammation and disease. The objectives of this study were to establish the identities of the isolates by comparison with characteristics listed in Bergey's Manual of Determinative Bacteriology (Holt, 1994), and to assess if lower airway disease was associated with a large or comparatively small number of species of this general group of bacteria.

Summary

Undifferentiated members of the *Actinobacillus/Pasteurella* group of bacteria were found to be significantly associated with lower airway disease in young training Thoroughbred horses (Wood *et al*, 1993). The identities of 71 isolates from 65 horses with or without evidence of lower airway disease, were determined to assess if the association with disease was accounted for by a small or large number of species. Fifty-two percent were *Actinobacillus equuli*, 18.3% were *Actinobacillus suis* -like, 12.7% were *Pasteurella pneumotropica*, 8.5% were *Actinobacillus ligniersii*, 7.0% were *Pasteurella haemolytica* and 5.6% were *P. mairii*. These results suggest that a range of *Actinobacillus/Pasteurella* species can be isolated from the lower airways of horses, but greater than half of the isolates are *A. equuli*. Lower airway inflammation was significantly associated with *A. suis* -like bacteria and *A. ligniersii*, but association was unclear for *A. equuli*. A much larger sample would be required for statistical purposes.

Materials and methods

Sources of the isolates

Details of horses are given in Table 1. Samples were taken from horses kept at nineteen different yards around England. Eleven of the yards were in Suffolk, two each were in Berkshire, West Sussex and Yorkshire, and one each was in Norfolk and Lancashire. Fifty-five of the horses were Thoroughbred horses in training, four were hunter-types, one was a pony, one was a Thoroughbred horse at stud and four were of unknown breed. Each was assigned a number (1-65), and the yards from which they came were identified by a designated letter (A-U) and the county. Tracheal wash lavage samples were taken and processed by the methods of Whitwell and Greet (1984). Briefly, the tip of an endoscope was introduced via the ventral nasal meatus, then glottis, and guided into the tracheal until the carina could be seen. A sterile plastic catheter was placed into the biopsy channel of the endoscope and 30ml of sterile phosphate buffered saline was squirted through this using a syringe. The fluid pooled at the thoracic inlet (which is the lowest point of the trachea in a horse standing with its head up), from where it was sucked back into the syringe via the catheter. In between horses, the endoscope was disinfected and sterilized at the end of the day. The sample was divided into three; the first aliquot was put into a sterile container for bacteriology, the second into an equal volume of 10% formalin for differential cytology and the third into an EDTA tube for a total nucleated cell count. The inflammation score comprised three equally contributing parameters, each adding one unit to the score. One unit was added to the score for each of the following: 1) if the count per millilitre of nucleated cells was greater than 1000. 2) if the degree of mucopus visible on endoscopy was moderate or greater and 3) if neutrophils were present in moderate or greater proportions relative to other cell types.

Identification tests

Isolates from fresh samples were identified upon the third passage and isolates processed before October 1993 were revived from storage at -50°C, necessitating two additional passages. Colonies were inspected after 3 passages on Wilkins-Chalgren agar (Unipath) supplemented with 5% citrated horse blood, or heparinised sheep blood. Note was made

of colony morphology and in particular of any haemolysis, or stickiness when picked up with a loop. Smears were made onto glass slides, heat fixed, Gram stained and examined under the microscope with x100 objective lens under oil immersion. One colony of each isolate was inoculated into a separate 10ml Tryptic soya broth (Difco) and incubated for 18 hours at 37°C, the ten drops of each (approximately 1ml) were added to broths containing different sugars. The fermentation of lactose, salicin, sorbitol, trehalose, or melibiose was tested using the medium described by Thompson and Knudsen (1958), quoted by Cowan and Steel (1993). These were incubated at 37°C and inspected after 24 and 48 hours. A positive result was recorded when the broth changed to a yellow colouration and a negative result if the broth remained orange/red. A delayed result was recorded if a particular broth was negative upon inspection after 24 hours, but positive after 48 hours. After incubation, sugar and peptone waters were sub-inoculated onto blood agar plates incubated at 37°C for 48 hours at each stage to test for purity. Tests for growth on MacConkey agar and production of indole, catalase and oxidase were as described by Cowan and Steel (1993). Physical, biochemical and cultural characteristics of the isolates were compared with those listed in Bergey's Manual of Determinative Bacteriology (Holt, 1994).

Type strains from the National Collection of Type Cultures (NCTC) were used as references for identification tests. These were *A. equuli* NCTC 8529, *A. ligniersii* NCTC 4189, *P. haemolytica* serotype 2 NCTC 9380, *P. mairii* NCTC 10699, and *P. pneumotropica* NCTC 8141. No type strain was available for *A. suis* so *A. suis* NCTC 12182, from a human wound from a horse bite was used.

Statistical analyses

One objective was to identify the isolates to find evidence of subtypes behaving epidemiologically as though they are primary pathogens. The testing of whether each species was more common amongst cases positive for Actinobacillus/Pasteurella bacteria compared with controls was analyzed by Chi-square using Epi info (Dean *et al.*, 1990).

Such evidence might be taken as support for a subtype which was causing disease more frequently than not when it is present.

Results and Discussion

The colony morphology of all isolates was very similar; they were grey and up to 3 millimetres in diameter. However, some colonies were stickier to pick up with a loop than others; those ultimately identified as *A. equuli* were particularly sticky. All of the isolates and most of the type strains were weakly haemolytic on Wilkins Chalgren agar (Unipath Ltd), with 5% citrated horse blood added, but isolates eventually identified as *A. equuli*, *A. suis*, or *A. ligniersii*, plus their NCTC strains, were weakly haemolytic on sheep blood agar, whilst *P. pneumotropica* and *P. mairii* were not. Colonies of the *P. haemolytica* type strain were beta haemolytic on both types of blood agar. The differential properties of all test and NCTC isolates are shown in Table 2. Isolates produced pink, lactose fermenting, colonies on MacConkey agar, with the exception of half of those identified as *P. pneumotropica*, (excluding the type strain of *P. pneumotropica*). Microscopically, isolates from solid and broth cultures were Gram negative and pleiomorphic, with evidence of bipolar staining. All of the isolates were oxidase and catalase positive. The results of carbohydrate fermentation identified 37 isolates from 34 horses and one pony as *A. equuli*, based on fermentation of lactose, trehalose and melibiose, and failure to ferment salicin. Of these, 22 fermented sorbitol like the type strain, and 12 did not. Three further isolates fermented sorbitol after a delay of approximately 48 hours. All were urease positive, but negative for indole production.

There were 13 *A. suis* isolates from 12 horses, which fermented lactose, salicin, trehalose and melibiose, but did not ferment sorbitol. All were urease positive and indole negative. The *A. suis* NCTC strain had the same properties. These fermented L- arabinose so may not be identical to Bisgaard Taxon 11, which is a previously described equine isolate (Bisgaard et al., 1984).

Eight isolates from 8 horses were identified as *P. pneumotropica*. These fermented lactose, but did not ferment salicin or sorbitol. Half fermented trehalose and six of the eight fermented melibiose. The type strain of *P. pneumotropica* had similar properties, except

that it fermented salicin; it also fermented trehalose and melibiose. All were positive for urease and indole production.

Six isolates from 6 horses were *A. ligniersii*. These fermented lactose and sorbitol, but did not ferment salicin, trehalose or melibiose. The type strain of *A. ligniersii* had similar properties, but it did not ferment sorbitol. All isolates were urease positive, but negative for indole production.

Five isolates from 5 horses were *P. haemolytica*. These fermented lactose, sorbitol and melibiose, but did not ferment salicin or trehalose. All five isolates were urease negative and negative for indole production. The type strain of *P. haemolytica* had similar properties, but it fermented salicin yet not lactose. Four isolates from 4 horses were *P. mairii*. These fermented trehalose, and sorbitol at 48 hours incubation, but did not ferment salicin and only one isolate fermented melibiose. The type strain behaved similarly, but did not ferment lactose and fermented salicin. Half fermented lactose and all were positive for urease, but negative for indole production.

Actinobacillus/ Pasteurella species were isolated from six horses on more than one occasion. Sorbitol fermenting *A. equuli* was isolated from horse 24 (yard P in Suffolk) on two occasions, two months apart. *A. equuli*, which did not ferment sorbitol, was isolated from horse 60 (yard S, also in Suffolk) but *A. equuli* that fermented sorbitol was isolated from this animal two months later. *A. suis* was isolated from horse 19 (yard L in Suffolk) for two consecutive sampling dates one month apart. *P. mairii* was isolated from horse 48 (yard S) one month after isolating *P. pneumotropica*. *P. pneumotropica* was isolated from horse 18 (yard F, Berkshire) five months after *A. ligniersii* was isolated. *P. pneumotropica* was isolated from horse 42 (yard R, Suffolk) one month after *P. haemolytica* was isolated.

Typically of bacterial lower airway infection, overt signs of disease were not evident by superficial examination of the horse at rest in the majority of cases and was only detected by endoscopy (Wood *et al.* 1993). Seven had a cough (horses 3,4,5,17,19,20 and 57). The

inflammation score of five of these was recorded as 3/3. Horse 21 had respiratory distress and an inflammation score of 2/3. Five of these horses yielded *A. equuli* (3,4,20, 21 and 57) and the other three yielded *A. suis* -like isolates (5,17 and 19).

Inflammation scores, where given, varied between the minimum (0/3) and maximum (3/3). Analysis of whether the different species were more common amongst horses with a score of 1 or more, revealed an association of cases for *A. suis* -like isolates (OR = 11.68; 95%CI; 0.64-210; $p = 0.026$) and *A. ligniersii* (OR = 11.00; 95%CI; 1.18-102; $p = 0.022$). In contrast, *P.pneumotropica* was associated with horses with a tracheal pathology score of 0 (OR = 0.19; 95%CI; 0.04-0.91; $p = 0.04$). There was no clear association with *A. equuli*. These statistical tests should be interpreted with some caution, given the small number of animals involved, but they suggest a stronger pathogenic role for *A. suis* -like isolates and *A. ligniersii*. Given the greater prevalence of *A. equuli* and the overall association of the Actinobacillus/Pasteurella group of bacteria with bacterial lower airway disease, it is still probable that *A. equuli* would be associated with disease when all animals are included in an analysis (i.e. including those animals without the Actinobacillus/Pasteurella group of bacteria).

Table 1. Actinobacillus/Pasteurella species identified from tracheal wash samples. (N/A = data not available).

Sampling date	Horse number	Yard and county	Inflammation score	Species identification
May 1992	1	A, West sussex	N/A	<i>A. equuli</i>
December 1992	2	B, Suffolk	N/A	<i>A. equuli</i>
December 1992	3	C, Suffolk	3/3	<i>A. equuli</i>
February 1993	4	D, West sussex	N/A	<i>A. equuli</i>
March 1993	5	E, Suffolk	3/3	<i>A. suis</i>
April 1993	6	F, Berkshire	1/2	<i>A. ligniersii</i>
June 1993	7	G, Suffolk	2/2	<i>P. haemolytica</i>
July 1993	8	H, Berkshire	N/A	<i>P. haemolytica</i>
October 1993	9	J, Suffolk	1/3	<i>A. equuli</i>
November 1993	10	K, Norfolk	N/A	<i>A. equuli</i>
December 1993	11	E, Suffolk	N/A	<i>A. equuli</i>
December 1993	12	J, Suffolk	2/2	<i>A. equuli</i>
December 1993	13	J, Suffolk	3/3	<i>A. suis</i>
December 1993	14	F, Berkshire	N/A	<i>A. equuli</i>
December 1993	15	E, Suffolk	3/3	<i>A. equuli</i>
January 1994	16	F, Berkshire	2/2	<i>A. equuli</i>
January 1994	17	J, Suffolk	3/3	<i>A. suis</i>
January 1994	20	J, Suffolk	N/A	<i>A. equuli</i>
February 1994	18	F, Berkshire	3/3	<i>A. ligniersii</i>
February 1994	19	L, Suffolk	3/3	<i>A. suis</i>
February 1994	21	M, W. Yorkshire	2/3	<i>A. equuli</i>
March 1994	22	J, Suffolk	1/3	<i>A. suis</i>
March 1994	23	N, Suffolk	N/A	<i>A. equuli</i>
March 1994	24	P, Suffolk	0/3	<i>A. equuli</i>
March 1994	25	J, Suffolk	3/3	<i>A. equuli</i>
March 1994	19	L, Suffolk	1/3	<i>A. suis</i>
March 1994	26	J, Suffolk	1/3	<i>A. equuli</i>
March 1994	27	J, Suffolk	3/3	<i>A. equuli</i>
April 1994	28	F, Berkshire	N/A	<i>P. haemolytica</i>
April 1994	29	J, Suffolk	N/A	<i>A. suis</i>
April 1994	30	Q, Lancashire	2/2	<i>A. equuli</i>
April 1994	31	E, Suffolk	0/3	<i>A. equuli</i>
May 1994	32	P, Suffolk	0/3	<i>A. equuli</i>
May 1994	24	P, Suffolk	0/3	<i>A. equuli</i>
May 1994	33	P, Suffolk	0/3	<i>P. mairii</i>
May 1994	34	P, Suffolk	2/3	<i>A. equuli</i>
May 1994	35	P, Suffolk	1/3	<i>A. equuli</i>
May 1994	36	J, Suffolk	N/A	<i>A. equuli</i>
June 1994	37	F, Berkshire	N/A	<i>P. mairii</i>
June 1994	38	P, Suffolk	3/3	<i>A. suis</i>
June 1994	39	P, Suffolk	0/3	<i>A. equuli</i>
June 1994	40	P, Suffolk	2/3	<i>A. suis</i>
July 1994	18	F, Berkshire	0/3	<i>P. pneumotropica</i>
July 1994	41	F, Berkshire	2/3	<i>P. pneumotropica</i>
August 1994	42	R, Suffolk	0/3	<i>P. haemolytica</i>
August 1994	43	S, Suffolk	0/3	<i>A. equuli</i> + <i>A. ligniersii</i>
September 1994	44	R, Suffolk	1/3	<i>A. equuli</i>
September 1994	42	R, Suffolk	0/3	<i>P. pneumotropica</i>
September 1994	45	S, Suffolk	0/3	<i>A. equuli</i>

Table 1. *Actinobacillus/Pasteurella* species identified from tracheal wash samples. (N/A = data not available).

Month / Year	Horse number	Yard and county	Inflammation score	Species
October 1994	46	S, Suffolk	1/3	<i>P. haemolytica</i>
October 1994	47	S, Suffolk	1/3	<i>A. ligniersii</i>
October 1994	48	S, Suffolk	0/3	<i>P. pneumotropica</i>
October 1994	49	S, Suffolk	1/3	<i>P. mairii</i>
November 1994	48	S, Suffolk	0/3	<i>P. mairii</i>
November 1994	50	S, Suffolk	1/2	<i>A. suis</i>
November 1994	51	S, Suffolk	1/3	<i>A. ligniersii</i>
November 1994	52	S, Suffolk	1/3	<i>A. ligniersii</i>
January 1995	53	F, Berkshire	0/3	<i>P. pneumotropica</i>
January 1995	54	F, Berkshire	1/3	<i>P. pneumo. + A. suis</i>
January 1995	55	P, Suffolk	0/3	<i>P. pneumotropica</i>
February 1995	56	J, Suffolk	N/A	<i>A. equuli</i>
February 1995	57	S, Suffolk	3/3	<i>A. equuli</i>
May 1995	58	J, Suffolk	N/A	<i>A. equuli</i>
May 1995	59	J, Suffolk	N/A	<i>A. equuli</i>
September 1995	60	S, Suffolk	0/3	<i>A. equuli</i>
November 1995	61	R, Suffolk	1/3	<i>A. suis</i>
November 1995	60	S, Suffolk	2/3	<i>A. equuli</i>
January 1996	62	P, Suffolk	1/3	<i>P. pneumotropica</i>
April 1996	63	T, Suffolk	N/A	<i>A. equuli</i>
April 1996	64	T, Suffolk	3/3	<i>A. equuli</i>
September 1996	65	U, W. Yorkshire	N/A	<i>A. suis</i>

Table 2. Differential properties of 71 isolates of *Actinobacillus/Pasteurella*-like bacteria from the equine lower airway, and NCTC strains (shown as numbers of isolates giving a positive reaction; L = fermentation positive only after 48 hours).

	<i>A. equuli</i> n = 37 (1)	<i>A. suis</i> n = 13 (1)	<i>P. haemolytica</i> n = 5 (1)	<i>P. pneumotropica</i> n = 8 (1)	<i>A. ligniersii</i> n = 6 (1)	<i>P. mairii</i> n = 4 (1)
No. of isolates	37 (1)	13 (1)	5 (1)	4 (1)	6 (1)	4 (1)
MacConkey growth	0 (0)	0 (0)	0 (0)	8 (1)	0 (0)	0 (0)
Indole	37 (1)	13 (1)	0 (0)	8 (1)	6 (1)	4 (1)
Urease						
Fermentation of:						
Lactose	37 (1)	13 (1)	5 (0)	8 (1)	6 (1)	2 (0)
Salicin	0 (0)	13 (1)	0 (1)	0 (1)	0 (0)	0 (0)
Sorbitol	22 + 3L (1)	0 (0)	5 (1)	0 (0)	6 (0)	4L (1)
Trehalose	37 (1)	13 (1)	0 (0)	4 (1)	0 (0)	4 (0)
Melibiose	37 (1)	13 (1)	5 (1)	6 (1)	0 (0)	1 (0)

3.0 CHAPTER THREE

UTILISATION AND BINDING OF EQUINE TRANSFERRIN BY ACTINOBACILLUS EQUULI AND CHARACTERISATION OF ITS IRON REGULATED PROTEINS.

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Objectives

Four tracheal wash isolates of *A. equuli* from horses with lower airway disease, from geographically and temporally separate outbreaks, were selected : a) to test for interactions with host iron carrier proteins; b) to look for production of IRPs; and c) if IRPs were detected, to ascertain their antigenic diversity.

Summary

Four isolates of *Actinobacillus equuli*, cultured from tracheal lavage samples taken from horses in separate outbreaks of lower airway disease, were examined for their ability to use iron carried by different iron carrying proteins. Cultures were inoculated onto medium containing just enough iron chelator to inhibit growth. Growth of all four isolates was restored by the addition of equine holotransferrin, but not by the addition of equine apo-transferrin, haemin, myoglobin, cytochrome C, ferritin, haemoglobin, rabbit holotransferrin, or human holotransferrin. Binding of equine holotransferrin was detected in all isolates cultured in iron limited media by probing colony blots with anti equine transferrin conjugated to horseradish peroxidase. None of the isolates bound any of the iron carrying compounds when they had been grown in iron replete conditions. Sodium salicylate extracts of the isolates grown in iron limited conditions analyzed by SDS-PAGE, contained proteins that were not present in extracts of iron replete cultures. Seven so called iron regulated proteins (IRPs) of different molecular masses, were present in all of the isolates. Major constitutively produced proteins of all of the isolates were similar in molecular mass and were antigenically related in immunoblots of SDS-PAGE gels incubated with rabbit antisera to sodium salicylate extracts of iron limited cultures. In contrast, there were varying degrees of antigenic cross-reactivity between the IRPs. Sera from young ponies geographically isolated from other equids, showed more selective reactivity for IRPs than rabbit antisera. Together, these results suggested a spectrum of IRP antigenic relatedness that was more sharply defined in the responses of horses to natural infection. Binding of equine holotransferrin by inducible iron regulated proteins may represent part of the mechanism by which *A. equuli* obtains iron essential for growth *in vivo*.

Materials and methods

Bacteria

A. equuli isolated from the tracheal washes of four horses with evidence of lower airway disease, supplied by the Animal Health Trust's (Newmarket,UK.) diagnostic laboratory, were labeled R, S, X and Y. R came from a Thoroughbred horse trained in Newmarket, Suffolk in December 1992; S came from a horse in a different training yard in Newmarket in October 1993; X came from a Thoroughbred horse trained in Lambourn, Berkshire, in December 1993; Y was from a hunter type kept in work at the Animal Health Trust, Newmarket, in December 1993. All were present in tracheal washes in numbers greater than 10^5 cfu / ml in association with mucopus and large numbers of polymorphonuclear leukocytes. Isolates were identified as *A. equuli* using methods described previously in Chapter 2.

Culture media

Isolates were stored at -70°C, revived and maintained as described in Chapter 2. TSB with added 2,2-dipyridyl (Sigma) was used to produce iron limited broth cultures. For the iron compound utilization assay, tryptic soya agar (TSA) was prepared by adding 5mg/ml bacteriological agar (Unipath Ltd), to TSB containing 2,2-dipyridyl at various concentrations specified below. All incubations were at 37°C unless stated otherwise and blood agar plates were incubated in the presence of 5% carbon dioxide, in air.

Determination of conditions for iron limited culture and iron compound utilization assay.

An 18 hour static culture of each isolate was added to prewarmed broths (37°C) without or with 2,2-dipyridyl (50 - 500µM) and incubated with shaking at 150rpm. After 4 hours, when turbid to the eye, the optical density (OD₆₀₀) was measured at 30 minute intervals. Once cultures had stopped growing, on the basis of optical density measurement, viable counts were made by counting the colonies resulting from spread inoculating 100µl of culture serially diluted in 150 mM phosphate buffered saline, pH 7.2 (PBS), onto horse blood agar, which were incubated for 18 hours. A concentration of 170µM 2,2-dipyridyl was sufficient to reduce the growth of all isolates by 10-fold in TSB. The concentration of

2,2-dipyridyl for the iron utilization assay was chosen by spreading 100 μ l of an 8 hour static culture of each isolate on a separate set of TSA plates made with a range of 2,2-dipyridyl concentrations, from 50 - 1000 μ M. A concentration of 300 μ M was just sufficient to completely inhibit growth on TSA without a supplementary iron source.

Iron compound utilization assay

A 100 μ l aliquot of an 8 hour static culture of each isolate (TSB without added 2,2-dipyridyl) was spread over the surface of TSA containing 300 μ M 2,2-dipyridyl and wells of 5mm diameter were aseptically punched into the agar. 10 μ l of a 10 mg / ml solution of each of the iron carrying compounds mixed with phosphate buffered saline was added to an individual well in the agar plate. Equine haemin, myoglobin, cytochrome C, ferritin, apo-transferrin, haemoglobin, human holo-transferrin and rabbit holo-transferrin were supplied by Sigma. The equine holotransferrin was prepared by modification of a previously described method (Caldwell and Archibald, 1987). Briefly, 10mg/ml apo-transferrin was mixed for 1 hour with 0.1% (w/v) ferric ammonium citrate dissolved in 40mM tris-bicarbonate buffer, pH 7.4, followed by dialysis with 3 changes of 1 litre 40mM tris-bicarbonate buffer, pH 7.4, over 3 hours, followed by 4 changes of 3 litre PBS over 4 hours. Equine haemoglobin was prepared fresh each time. Ferric ammonium citrate at 0.1M concentration, 0.1M ferrous sulphate, 40mM tris-bicarbonate buffer, pH 7.4, and PBS, were also added to separate wells on each plate as positive and negative controls. The plates were incubated lids uppermost for 24 hours, after which areas of growth around the wells were recorded.

Growth of isolates in iron limited and replete media

A 50ml, TSB culture of each isolate grown statically for 18 hours, was used to inoculate a sterile fermentation vessel of 800 ml TSB containing 170 μ M 2,2-dipyridyl. A separate vessel containing TSB without 2,2-dipyridyl was similarly treated. The vessels were kept at 37°C and the dissolved oxygen was adjusted to 30% by a combination of different rates of oxygen supplementation and stirring. Optical density readings (OD₆₀₀) were taken hourly and both pH and dissolved oxygen recorded automatically at ten minute intervals.

When these values no longer changed, the cultures were refrigerated at 4°C overnight. The viable and total bacterial cell count was determined using aliquots from each fermentation. Cultures were centrifuged at 10,000g and the supernatant separated off and freeze dried. The pellet was resuspended in 8ml PBS and freeze dried.

Transferrin binding assay

Four *A. equuli* isolates grown with the concentration of 2,2-dipyridyl which reduced growth ten fold for each isolate (170µM), or grown without 2,2-dipyridyl, were freeze dried and sodium salicylate extracts made of each (see below for preparation). Cells of each isolate were resuspended to total counts of 10^8 , 10^4 and 10^2 , and along with their culture supernatants, were separately transferred onto nitrocellulose (Schleicher and Schuell) using a BioDot apparatus (BioRad) according to the manufacturer's instructions. Ten micrograms of sodium salicylate extract of each isolate, were applied to three separate wells as a 50 µg / ml solution in; a) PBS, b) PBS containing 0.2% (w/v) SDS, or c) PBS containing 0.2% (v/v) 2-mercaptoethanol. Blots were shaken on a rocking platform throughout at room temperature (approximately 20°C). Unreacted areas of the dot blots were blocked with 5% (w/v) skimmed milk powder dissolved in 0.1% (v/v) Tween 20 in PBS (PBS-T) for one hour. After washing in three changes of PBS-T, once for 15 minutes then twice for five minutes, dot blots were mixed with 10µg/ml equine holotransferrin in PBS-T for 1 hour. Washing was repeated in PBS-T as before, after which horseradish peroxidase (HRP) conjugated sheep anti-horse transferrin antibody (Cambio) diluted 1:3000 in PBS-T was added for 1 hour. After rewashing in five changes of PBS-T, once for 15 minutes then four times for five minutes, cells binding transferrin and hence HRP conjugated anti-transferrin, were detected using the Enhanced Chemiluminescence detection system (ECL, Amersham).

Preparation of sodium salicylate extracts

Freeze dried cultures grown with or without 170µM 2,2-dipyridyl were resuspended in 1M sodium salicylate and shaken at 37°C for 3 hours. The supernatant fraction was collected after centrifugation at 3000g for 40 minutes at 4°C and dialyzed in two changes of 3 litres

PBS over 24 hours, followed by distilled water for 18 hours. The protein concentration of the extracts were measured using a bicinchoninic acid assay (Pierce).

SDS- polyacrylamide gel electrophoresis

Sodium salicylate extracts of cells (equivalent to 25µg of protein) grown with or without 170µM 2,2-dipyridyl, and molecular mass markers (Sigma), boiled in Laemmli sample buffer containing marker dye for 2 minutes, were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (Laemmli, 1970), with a 12% (w/v) polyacrylamide separating gel and a 4% (w/v) stacking gel in a Protean II apparatus (BioRad) and a constant current of 24mA at room temperature for approximately 4 hours.

Proteins in the gel were detected by silver staining. Gels were serially incubated and shaken at room temperature a) in an aqueous mixture of 50% (w/v) ethanol and 10% (w/v) methanol for 30 minutes, b) in an aqueous mixture of 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, 33% (w/v) ethanol and 2% (w/v) glutaraldehyde for 30 minutes, c) in three water washes of 5 minutes each, d) in an aqueous mixture of 0.1% (w/v) silver nitrate and 0.0076% (w/v) formaldehyde, for 20 minutes, e) in water briefly, f) in an aqueous mixture of 0.23% (w/v) sodium carbonate containing 0.0003% (v/v) formaldehyde, until bands were visible without excess background, and finally g) in 1.5% (w/v) aqueous EDTA solution for ten minutes. Gels were soaked in 10% (v/v) glycerol for 15 minutes and dried between polythene (Pharmacia), on a gel drier (Biorad), for 2 hours at 80°C. Molecular masses of proteins were calculated by their relative mobility in relation to proteins of known molecular mass in the standards.

Native polyacrylamide gel electrophoresis

Sodium salicylate extracts of cells grown with or without 170µM 2,2-dipyridyl (equivalent to 25µg of protein), were mixed with Laemmli sample buffer without the SDS or 2-mercaptoethanol, and were separated by polyacrylamide gel electrophoresis (PAGE), without SDS, in a 12% (v/v) polyacrylamide separating gel and a 4% (v/v) stacking gel in a

Protean II apparatus (BioRad) and a constant current of 24mA at room temperature until the dye front reached the bottom of the gel. The gel was then electroblotted onto nitrocellulose (see below).

Serum samples

Each of four, six month old, specific pathogen free, female, New Zealand White rabbits (Charles River), were test bled and separately given 150 µg of dipyrindyl grown sodium salicylate extract from one of the four strains, mixed with Freund's Incomplete adjuvant (Sigma), by subcutaneous injection. Two subsequent monthly booster inoculations were given, but each extract was mixed with an equal volume of PBS without adjuvant. Rabbits were bled weekly and the sera were harvested; blood was kept at 4°C overnight to allow the clot to shrink and serum was removed and spun briefly to remove any red blood cells remaining. The antibody response was monitored by immunoblotting, as described below.

Sera from three groups of ponies were obtained. Group A were twenty-one Welsh Mountain ponies aged less than six months, with no known previous contact with other equines. These ponies were selected as those as young as were available in the hope that they had been infected with, and immunologically responded to a limited range of *A. equuli* proteins, so that the pattern of their reactivity with IRPs would be more likely to be restricted and discriminatory. Group B consisted of twelve ponies of approximately one year old kept at the Animal Health Trust, Newmarket, UK and not extensively mixed with other ponies. Group C consisted of eight ponies of approximately eighteen months old that had been extensively mixed with other ponies at the Animal Health Trust.

Western immunoblotting

Sodium salicylate extracts of each culture separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (Schleicher and Schuell) as previously described (Towbin *et al*, 1979), using a TransBlot wet blotting apparatus (BioRad; used according to the manufacturer's instructions) at 100V for 3 hours at 4°C. Blots were

shaken on a rocking platform throughout at room temperature, first in 5% (w/v) dried skimmed milk powder dissolved in PBS-T for one hour. After washing in three changes of PBS-T, once for 15 minutes then twice for five minutes, horse or rabbit antiserum diluted 1:100 in PBS-T was added, for 1 hour. After washing in PBS-T as described above, horseradish peroxidase (HRP) conjugated anti-horse or anti-rabbit immunoglobulin (Sigma) diluted 1:10 000 in PBS-T, was added for 1 hour. After washing in five changes of PBS-T, once for 15 minutes and four times for 5 minutes, proteins which bound serum antibody, and hence HRP conjugate were detected with Enhanced Chemiluminescence detection reagents (ECL, Amersham).

Examination of bacterial cell morphology and medium viscosity

Cultures of each isolate shaken at 150 rpm for 18 hours with or without 2,2-dipyridyl, were compared by the Gram staining technique. Viable counts were made for each culture, and aliquots were spun for 1 minute in a microfuge, then culture supernatants were quickly analyzed in a viscometer (Viscometer II; Coulter Diagnostics) and viscosity measurements were compared with those of sterile culture medium with or without 170 μ M 2,2-dipyridyl added.

Results and Discussion

Utilization of iron compounds for growth by *A. equuli*

All isolates of *A. equuli* grew around wells containing equine holotransferrin, ferric ammonium citrate and ferrous sulphate on medium containing enough 2,2-dipyridyl to completely inhibit growth without supplements, but not around wells containing equine apo-transferrin, haemin, myoglobin, cytochrome C, ferritin, or haemoglobin, or human holotransferrin or rabbit holotransferrin. Growth of all of the isolates on medium without 2,2-dipyridyl added was confluent.

Binding of holotransferrin by *A. equuli* cells and sodium salicylate extracts

Whole cells of each isolate grown in iron limited conditions and coated onto nitrocellulose, bound equine holotransferrin. However, when the *A. equuli* isolates were grown in iron replete medium, transferrin binding could not be detected for any of the isolates (Figure 1).

Sodium salicylate extracts of isolates S, X and Y grown in iron limited conditions tested in the same way also bound equine holotransferrin when mixed with PBS, or PBS containing 0.2% (w/v) 2-mercaptoethanol, but did not bind transferrin when mixed with PBS containing 0.1% (w/v) SDS. None of the extracts of isolates grown in iron replete medium were seen to bind transferrin and no binding ability could be detected by extracts of isolate R in any of the conditions tested. None of the resuspended culture media, or the sodium salicylate extracts separated by native PAGE and transferred to nitrocellulose, bound equine holotransferrin.

Proteins in extracts of isolates grown in iron limited and replete media

The distribution of proteins of different molecular mass in silver stained SDS-PAGE gels of extracts were complex but similar between isolates irrespective of whether they were grown in medium in which iron was replete or limited. All four isolates contained seven proteins in extracts of cultures grown in iron limited conditions which were absent or

much fainter in extracts of cultures grown in iron replete conditions (iron regulated proteins, IRPs). The molecular masses of these were approximately 99, 73, 69, 65, 60, 48 and 20.5 KDa (Figure 2 and Table 3).

The 99 KDa IRP was faintly detectable in the iron replete extracts of three of the isolates and the 73, 69 and 65 and 48 KDa IRPs were present faintly in all of the iron replete extracts. The 60, 20.5 and 20 KDa proteins could not be detected in the iron replete extracts. Polypeptides induced by the iron replete culture conditions were not detected. Constitutively produced proteins were of similar molecular mass for all four *A. equuli* isolates.

Antigenic relatedness of IRPs and other proteins characterized using immune rabbit antisera

Pre immune sera contained antibodies to some of the major non-IRPs detected in immunoblots of sodium salicylate extracts of both iron limited and replete cultures of the isolates R,S,X and Y (Table 4), suggesting that the rabbits had encountered similar determinants before and that there is a high degree of cross-reactivity between *A. equuli* and other bacteria colonizing rabbits.

Immunoblotting detected antibodies in immune sera to all of the R,S,X and Y IRPs seen in stained gels (99, 73, 69, 65, 60, 48 and 20.5 KDa) in homologous reactions to and in many heterologous reactions (Figures 3-6). An additional 36KDa protein, which could be an IRP was detected in all extracts, which was undetectable in stained SDS-PAGE gels, possibly because of co-migration with non-IRPs.

Some of the IRPs were not detected in some of the heterologous reactions, but were detected in homologous reactions, which indicated that failure to detect antibodies in heterologous antisera was due to antigenic differences between these proteins in the different isolates (Figures 3-6).

The IRPs of isolates R and X were the least cross-reactive, although each antiserum had cross-reactive antibody to one polypeptide of 48 KDa. The IRPs of R and S showed mutual strong antigenic cross-reactivity. The IRPs of Y showed strongest antigenic cross-reactivity to those of S. Many IRPs of S and Y reacted with antibody in antisera to R and X. However, the antibodies in antisera to S and Y had a more restricted pattern of reactivity for IRPs of isolates R and X.

Antigenic relatedness between IRPs and other proteins of isolates R,S,X and Y characterized using equine sera

Three separate sets of pony sera designated A, B and C, were tested for antibody to IRPs and non-IRPs in extracts of each isolate separated by SDS-PAGE and transferred to nitrocellulose (Figure 7). The pony sera were more discriminating than the rabbit sera and showed less cross-reactivity. There were however, antibodies in most sera to many of the non-IRPs in extracts of all of the isolates, and to the 36KDa IRP detected with immune rabbit antisera.

Antibodies in the sera of some group A ponies, reacted with IRPs in extracts of isolates X and Y. Most of the reactivity for the IRPs of isolate Y was for the 73 KDa polypeptide whereas for isolate X it was for polypeptides of 60 KDa and below. None of the group A sera had antibodies to the IRPs of extracts of R or S although rabbit antisera to IRPs in isolate Y were strongly cross-reactive for those in isolate S.

Antibodies in the sera of group B ponies also reacted with IRPs in extracts of isolates X and Y, but not R or S. There were only antibodies to the 48 KDa and 20.5 KDa IRPs in extracts of both X and Y isolates. Antibody in most of the sera reacted to some non-IRPs present in all four extracts.

Antibodies in all of the group C pony sera reacted with some IRPs in the extracts of all four isolates. The reactivity of antibodies in these sera to extract Y was for a broader range of IRPs than for isolate X, whose IRPs of only 60 KDa and below were detected.

Conversely, antibodies were only detected in the extract of isolate R to IRPs of 60 KDa and above. Antibodies in all sera reacted with a broad range of IRPs in the extract of isolate S. The pattern of reactivity to IRPs in extracts of isolates R and X were most different as antibodies in most sera detected IRPs of 60 KDa and above in extract of isolate R, but detected IRPs of 60 KDa and below in extract of X.

Comparative cell morphology and viscosity of *A. equuli* cultures grown in iron limited and replete media

Microscopic examination of all cultures revealed that bacteria grown in the presence of 2,2-dipyridyl appeared shorter and were more darkly stained by the safranin counterstain used in Gram staining, than those grown in the absence of 2,2-dipyridyl. There was no difference in the viscosity of TSB with or without 2,2-dipyridyl. The viscosity of the culture supernatants of both media increased upon inoculation and incubation in the case of all four isolates. However, the relative viscosity of culture supernatants containing 2,2-dipyridyl increased more than those without 2,2-dipyridyl (Figure 8). Interestingly, the supernatant viscosity diminished as the time after centrifuging the cultures was increased.

Table 3. Antibodies in the sera of rabbits immunized with R,S,X, and Y to iron regulated proteins (kDa) in sodium salicylate extracts of iron restricted *A. equuli* cultures .

Isolate **Rabbit sera (denoted by the A. equuli isolate each was subsequently immunized with).**

R	(R)	(S)		(X)		(Y)	
		45 35 33		(52 46) 45 (44) 37 35,		(46) 45 35 33	
S	(S)	26 23 (22) 21.5 18	21.5 18	21.5 18		21.5	
		(52) 45 (40) 35	(52) 45 (37) 35 33	(46) 45 (37) 33		(46) 45 (37) 35 33	
X	(X)	(30 26 23) 21.5 18	(23) 21.5 18	(23) 21.5 18		21.5 18	
		(66) 46 45 44 35 33	74 66 62 (52) 45 (44 37) 35-33	(80 74) 58 (44 37) 35		(74 66) 58 52 (37) 35 30	
Y	(Y)	(26) 23 22 21.5 (20) 18	26 23 22 21.5 18	26 21.5 18		(27) 26 (23) 21.5 18	
		(80) 74 (66) 58 (52) 45 35	80 74 58 52 46 45 40 35 33	80 46 45 37 35		37 35	
		(30 26 23 22) 21.5 (20) 18	30 26 23 22 21.5 20 18	23 21.5 18		26 21.5 18	

Table 4. Antibodies in pre-immune and immune rabbit sera that reacted with proteins (KDa) in sodium salicylate extracts of iron replete *A. equuli* isolates R,S,X,and Y.

Isolate	Antiserum from rabbits Immunized with (strain).			
	(R)	(S)	(X)	(Y)
R	99 73 69 55 60 48 20.5	99 69 65 60 48 20.5	48 20.5	48 20.5
S	99 73 69 55 60 48 20.5	99 73 69 65 60 48 20.5	99 73 69 65 60 48 20.5	99 73 69 65 48 20.5
X	48	73 69 65 48	73 69 65 48	48
Y	99 69 65 60 48 20.5	99 73 69 65 60 48 20.5	99 69 65 60 48 20.5	99 69 65 60 48

Polypeptides in brackets were detected only by immune sera.

Table 5: Viscosity (measured in g / ms²) of the culture supernatant of *A. equuli* isolates R, S, X and Y grown in iron limited conditions and in standard medium.

<i>A. equuli</i>	Viscosity (g / ms- 2) Standard medium (no 2,2- dipyridyl)		Mean	Iron limited medium (with 2,2-dipyridyl)		Mean
R	0.97	0.98	0.98	1.02	1.03	1.03
S	0.98	0.98	0.98	1.02	1.01	1.02
X	0.98	0.99	0.98	1	1	1
Y	0.98	0.98	0.98	1	1.01	1.01
Medium control	0.95	0.95	0.95	0.95	0.95	0.95

Culture supernatant viscosity increased in iron limited medium as compared with standard medium.

Figure 1. Binding of equine holotransferrin by *A. equuli* isolates R, S, X and Y, grown in iron limited conditions and in standard medium. Columns 1,3,5 and 7 show isolates R, S, X and Y respectively, grown in standard medium. Columns 2,4,6 and 8 show isolates R, S, X and Y respectively, grown in iron limited medium. The left hand column shows the number of cells comprising each dot, in duplicate.

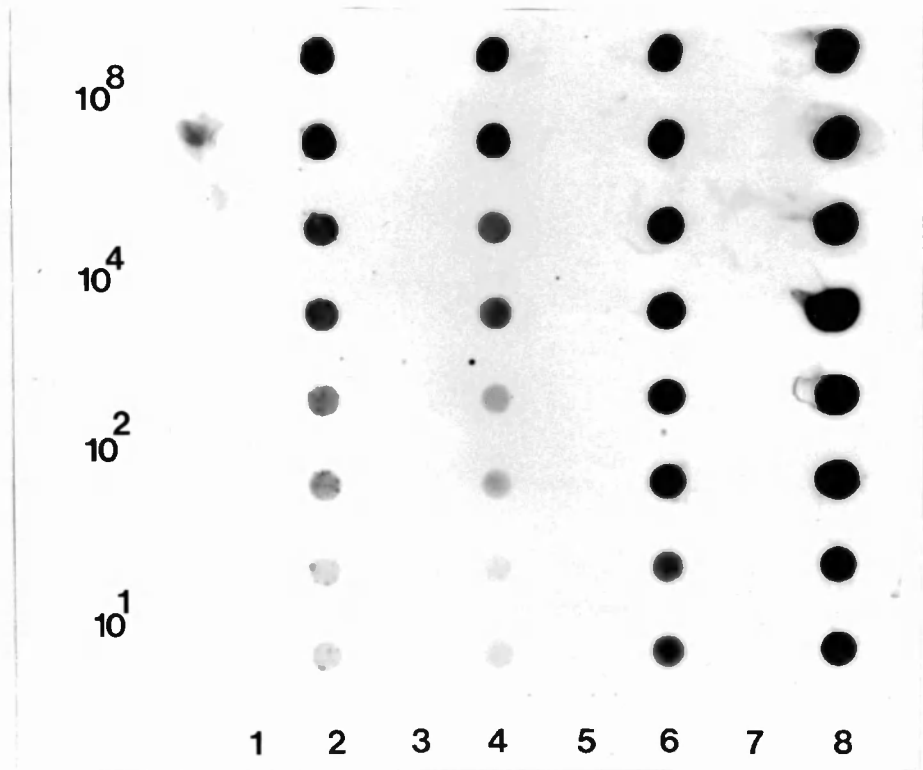


Figure 2. Silver stained SDS-PAGE of sodium salicylate extracts of *A. equuli* isolates R, S, X and Y, grown in iron limited and standard culture media. Lanes 2,4,6 and 8 show extracts of isolates R, S, X and Y respectively, grown in standard medium. Lanes 3,5,7 and 9 show extracts of isolates R, S, X and Y respectively, grown in iron limited medium. Lanes 1 and 10 show high molecular weight and Dalton Mark VII-L standards (Sigma). IRPs are indicated by their molecular mass (KDa).

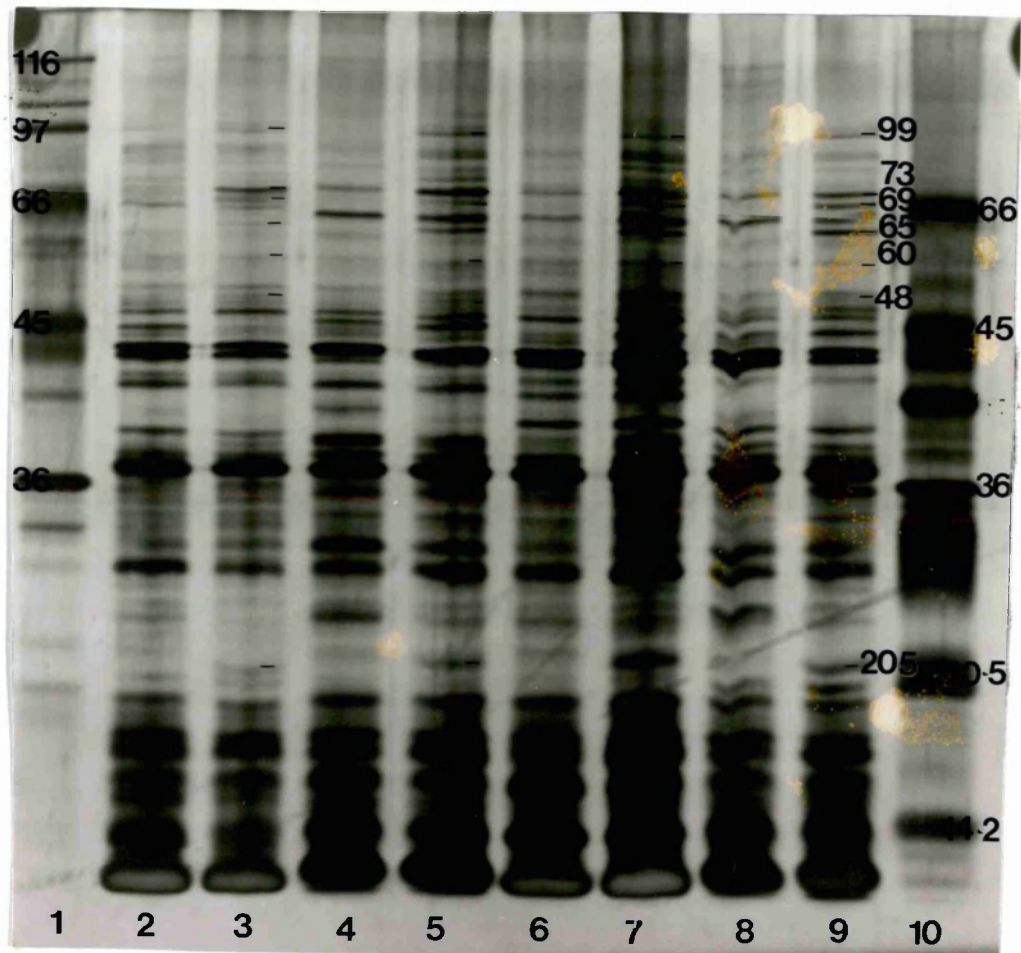


Figure 3. Immunoblot of rabbit antisera to sodium salicylate extracts of *A. equuli* isolates R, S, X and Y, with isolate R extract polypeptides (KDa), made from culture in iron limited and standard media. Lanes 1,3,5 and 7 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in standard medium. Lanes 2,4,6 and 8 show the reaction of antisera to extracts of isolates R, S, X and Y respectively, grown in iron limited medium.

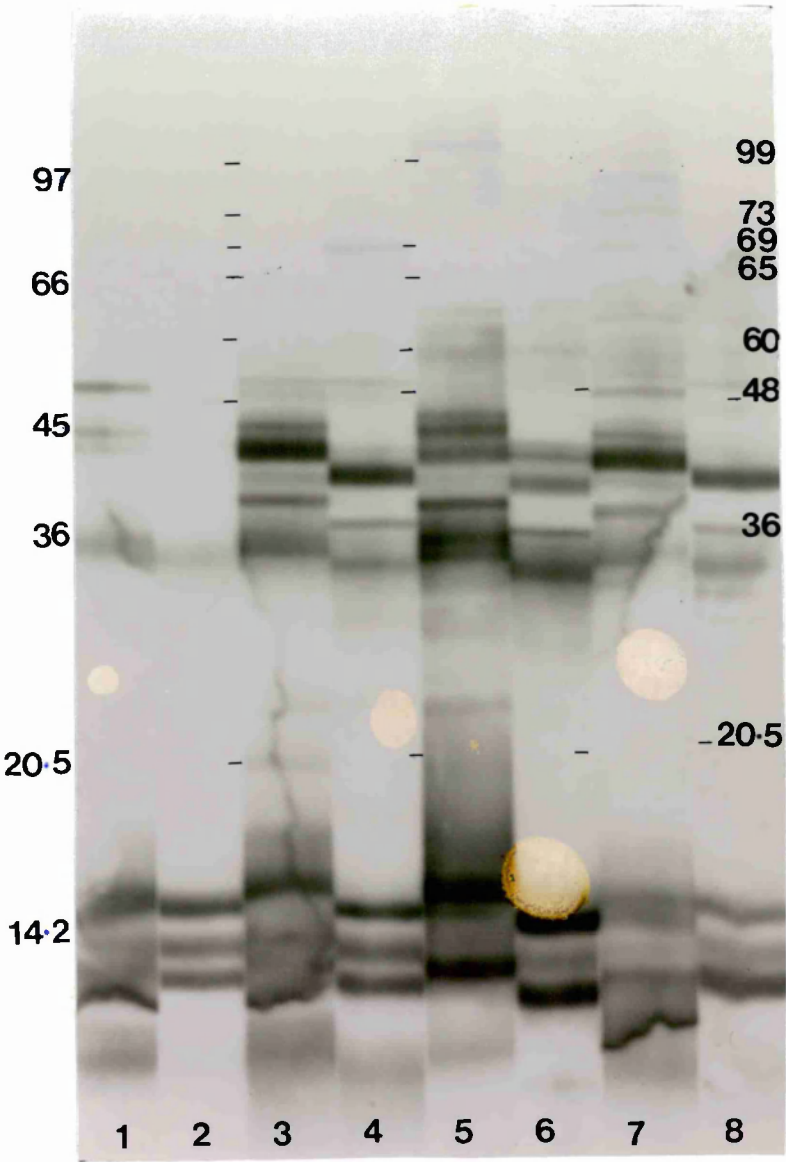


Figure 4. Immunoblot of rabbit antisera to sodium salicylate extracts of *A. equuli* isolates R, S, X and Y, with isolate S extract polypeptides (KDa), made from culture in iron limited and standard media. Lanes 1,3,5 and 7 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in standard medium. Lanes 2,4,6 and 8 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in iron limited medium.

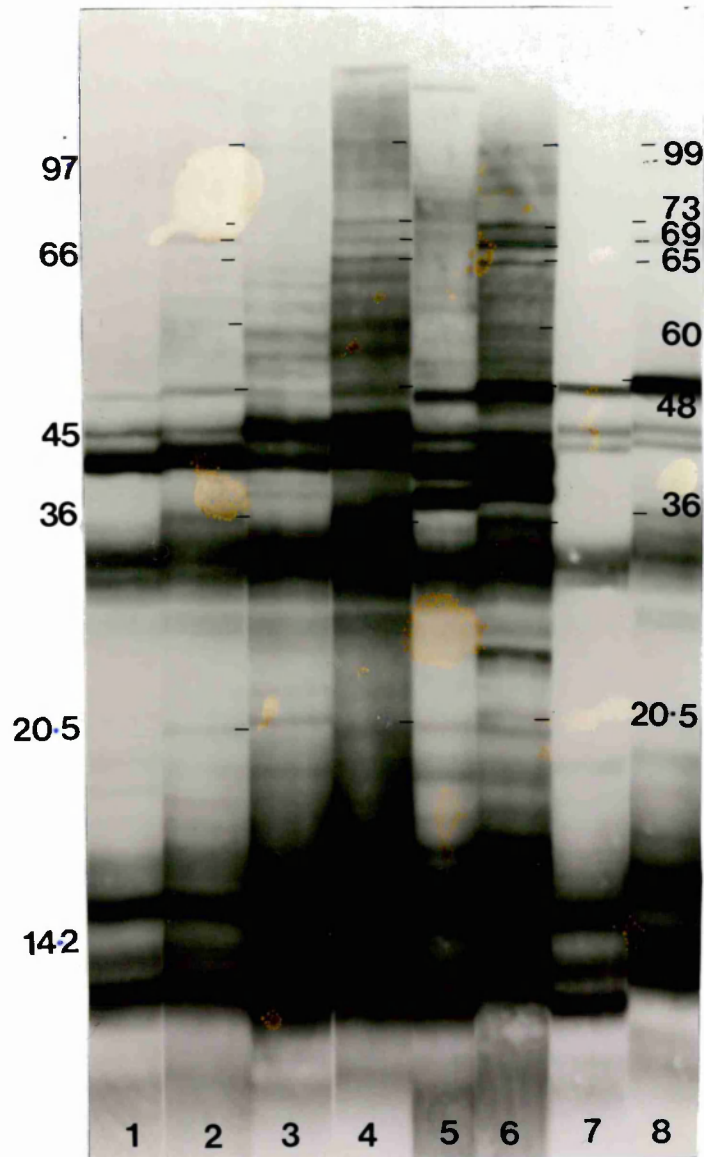


Figure 5. Immunoblot of rabbit antisera to sodium salicylate extracts of *A. equuli* isolates R, S, X and Y, with isolate X extract polypeptides (KDa), made from cultures in iron limited and standard media. Lanes 1,3,5 and 7 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in standard medium. Lanes 2,4,6 and 8 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in iron limited medium.

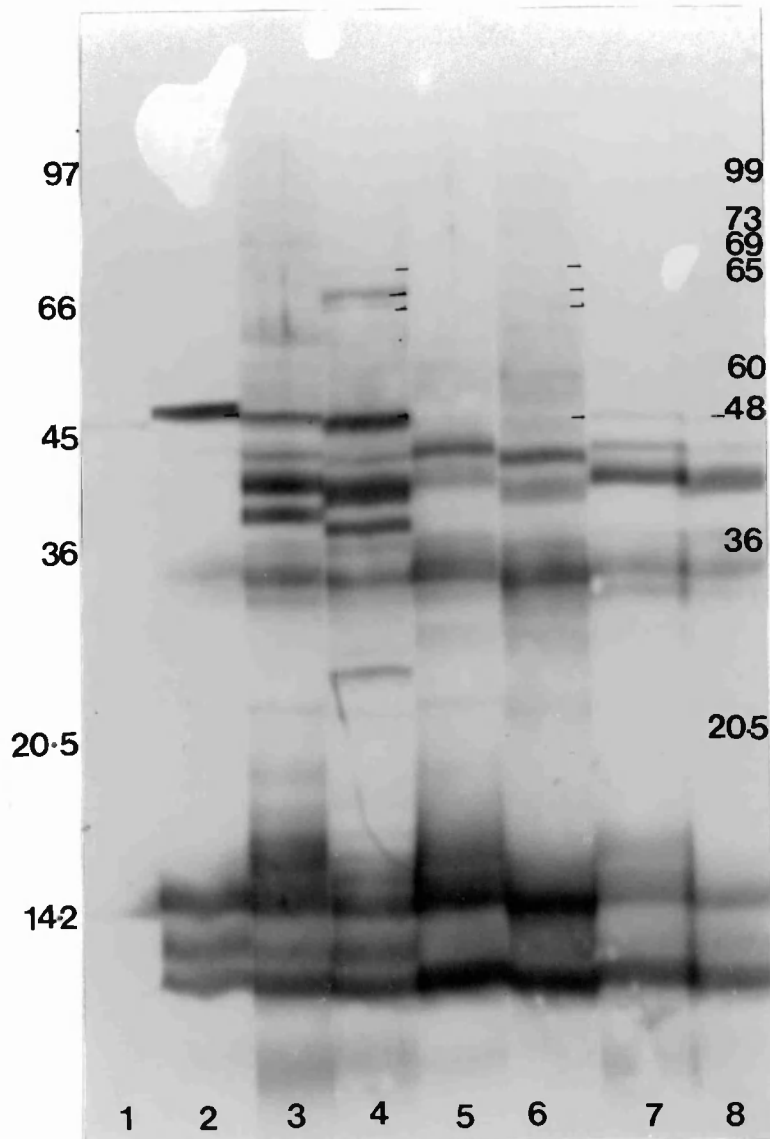


Figure 6. Immunoblot of rabbit antisera to sodium salicylate extracts of *A. equuli* isolates R, S, X and Y, with isolate Y extract polypeptides (KDa), with cultures in iron limited and standard media. Lanes 1,3,5 and 7 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in standard medium. Lanes 2,4,6 and 8 show the reactions of antisera to extracts of isolates R, S, X and Y respectively, grown in iron limited medium.

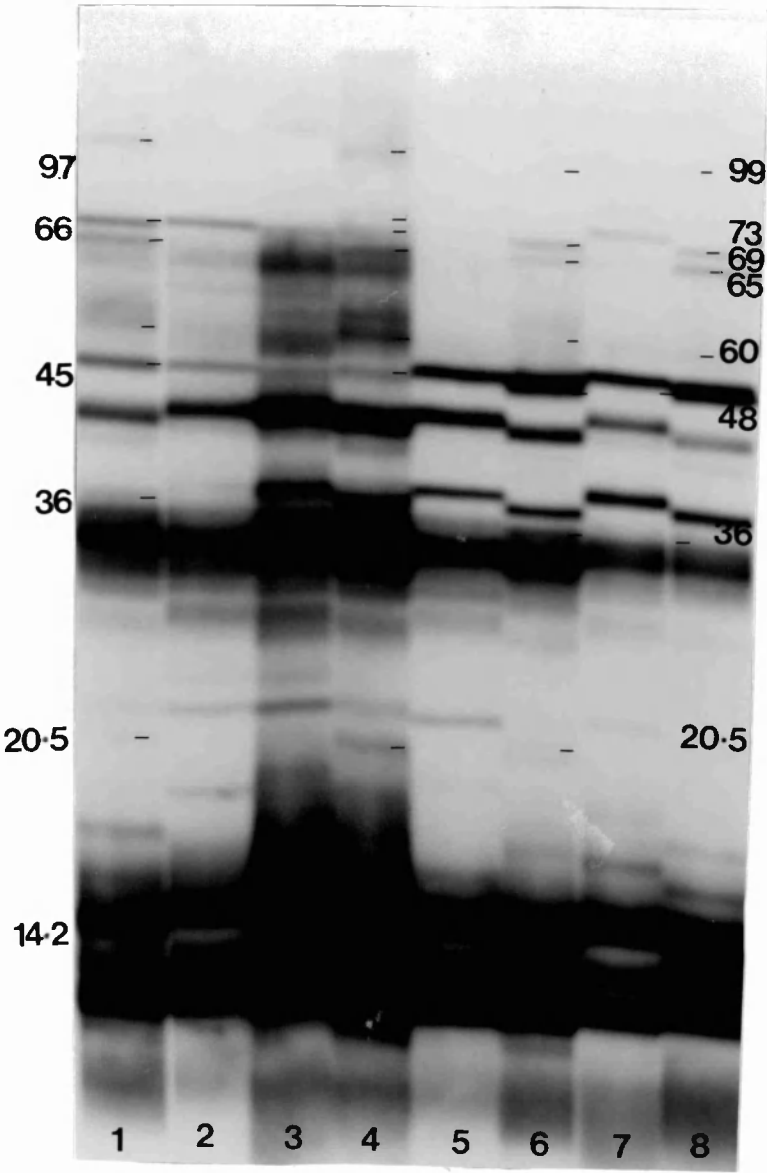
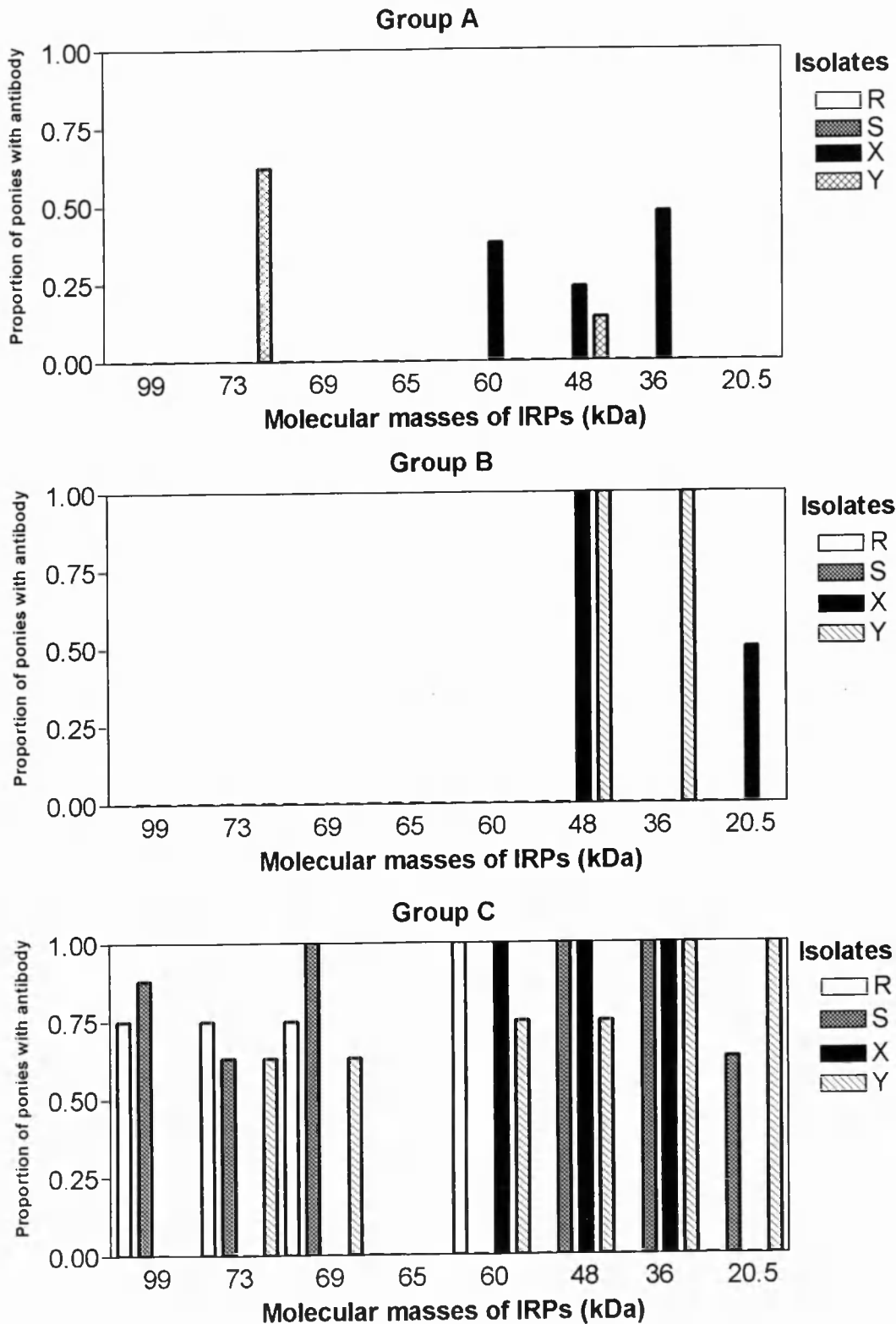


Figure 7. Iron restriction proteins (IRPs) of *Actinobacillus equuli* isolates R, S, X and Y, separated by SDS-PAGE and immunoblotted, detected by antibodies in the sera of pony groups A (< 6 months old) , B (12 months old) and C (18 months old)



4.0 CHAPTER FOUR

VARIATIONS IN THE ANTIGENICITY, OPSONOGENICITY AND
RESISTANCE TO PHAGOCYTOSIS OF DIFFERENT ISOLATES OF
STREPTOCOCCUS ZOOEPIDEMICUS FROM THE EQUINE TRACHEA.

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Objectives

The aims of the study were i) to investigate the antigenic relatedness between M-like proteins of the five distinct *S. zooepidemicus* isolates using immunoblotting and assays of opsonisation; ii) to develop a mouse model of challenge; iii) to assess the potential of bacterin immunization to protect against homologous challenge in a mouse model: iv) to assess the potential for cross-protection between isolates with the most dissimilar M-like proteins and opsonogenic determinants and v) to determine the relatedness of these isolates using the ribotyping scheme described by Chanter *et al*, (1996).

Summary

Five isolates of *Streptococcus zooepidemicus*, cultured from tracheal lavage samples taken from horses in separate outbreaks of lower airway disease, contained a similar polypeptide profile when hot acid extracts were analyzed by SDS-PAGE. These extracts are said to comprise mostly of M-like protein fragments, which are thought to be antiphagocytic. Immunoblots probed with rabbit antisera to the extracts of each isolate revealed a complex pattern of antigenic variation between the isolates, with some cross-reactivity. A ribotyping scheme based upon the 16S-23S RNA intergenic spacers (Chanter *et al.*, 1996) confirmed that these three were of the same ribotype, whilst the others were both different.

Each antiserum opsonised at least two of the isolates, in phagocytosis assays using rabbit blood as a source of polymorphonuclear leukocytes (PMN). A similar assay employing naturally occurring opsonic antibodies in ponies, using whole pony blood, suggested that there were no cross-reactive opsonins for all five *S. zooepidemicus* isolates. No relationship could be found between ribotype / antigenic profile and opsonogenic behavior, suggesting that M-like protein is not necessarily the principal anti-phagocytic mechanism for these isolates, at least *in vitro*.

Two of the isolates were selected for the greatest dissimilarity based on the results of immunoblots and phagocytosis assays to assess the potential for cross-protection in a mouse challenge model. Intranasal challenge of several inbred strains of mice was not a reliable respiratory model of infection. Intraperitoneal challenge however, was more reliable and was chosen to assess the effects of immunization. Immunization with formalin killed bacteria in Freund's incomplete adjuvant followed by homologous challenge significantly delayed the time to euthanasia *in extremis* against homologous infection, but too few mice survived to test any protective effect of immunization against heterologous challenge.

Analysis of a much greater number of isolates would be required to determine how many types of *S. zooepidemicus* should be included in an equine vaccine; this preliminary work

suggests that more than one type would be needed to provide protection against a broad range of isolates.

Materials and methods

Bacteria

S. zooepidemicus isolates from the tracheal washes of five horses with evidence of lower airway disease, supplied by the Animal Health Trust's (Newmarket, U.K.) diagnostic laboratory, were labeled A, B, C, D and E; A came from a Thoroughbred horse trained in Newmarket, Suffolk in November 1992; B came from a horse trained in Sussex in May 1992; C came from a Thoroughbred horse trained in Yorkshire, in November 1991; D was from a Thoroughbred horse trained in Lambourn, in May 1992. E came from the lung of a Welsh pony, examined post mortem, in June 1993. All were present in tracheal washes in numbers greater than 10^5 cfu / ml in association with mucopus and large numbers of polymorphonuclear leukocytes.

Colonies were beta haemolytic, mucoid, transparent, catalase negative, and of approximately 2mm in diameter. Gram staining revealed gram positive cocci of approximately 0.3-0.4µm diameter. One colony of each isolate was used to inoculate a 10ml THB, which was incubated statically for 18 hours, then ten drops of culture and four drops of horse serum (Unipath Ltd) were used to inoculate separate purple indicator broths (Purple broth base, Difco; made according to manufacturer's instructions) containing either salicin, sorbitol, lactose, or trehalose. After 24 hours incubation, the *S. zooepidemicus* isolates were positive (green/yellow colour) for lactose, salicin and sorbitol, but negative for trehalose (remained purple).

Culture media

Streptococci were stored at -50°C, revived and maintained as described for *Pasteurella* / *Actinobacillus* isolates in Chapter 2, but Todd Hewitt broth (THB; Unipath Ltd) was used in place of TSB for broth cultures. Incubations were at 37°C unless stated otherwise, and blood agar plates were incubated in the presence of 5% (v/v) carbon dioxide in air.

Preparation of M-like proteins

Methods reported by Boschwitz *et al.* (1991), were modified as follows: An 18 hour 10ml THB static culture incubated at 37°C for each isolate was inoculated into a separate 500ml THB and incubated at 37°C with continuous shaking. Bacteria were harvested by centrifugation at 10 000g (Sorvall RC-5B, GSA rotor) at 4°C for 20 minutes, then washed in PBS and centrifuged at 12 000g (Sorvall RC-5B, SS34 rotor) twice for thirty minutes. Cells were extracted in 1M hydrochloric acid at pH 2.5 and 100°C and neutralized after five minutes with 1M sodium hydroxide. Cells were pelleted by centrifugation at 12 000 g (Sorvall RC-5B, SS34 rotor) for 30 minutes. Supernatant fractions were dialyzed against two changes of 3 litres of PBS, at 4°C with stirring over 24 hours. The protein concentrations of the extracts were measured using a bicinchoninic acid assay (Pierce).

SDS-PAGE

Acid extracted M-like proteins (equivalent to 20µg of protein) were analyzed by SDS-PAGE and silver staining as described in Chapter 3.

Serum samples

Each of five six month old, specific pathogen free, female, New Zealand White rabbits (Charles River) were test bled, then separately immunized with 25µg of extract from one of the five isolates mixed with an equal volume of Freund's Incomplete adjuvant (Sigma), by subcutaneous injection. Three subsequent monthly booster inoculations were given, but each extract was mixed with an equal volume of PBS without adjuvant. Rabbits were bled weekly and the sera were harvested. The antibody response was monitored by immunoblotting, as described in Chapter 3.

The sera from three groups of young ponies named A, B and C, described in Chapter 3, were selected in the hope that they had been infected with, and immunologically responded to a restricted range of *S. zooepidemicus*, so that the pattern of their reactivity with M-like proteins of isolates would be restricted and discriminatory.

Western immunoblotting

Hot acid extracts separated by SDS-PAGE were transferred to nitrocellulose and tested for binding of antibody as described in Chapter 3.

Characterization of the *S. zooepidemicus* isolates using rabbit opsonic antisera and equine whole blood

Rabbit antisera were tested for their ability to separately opsonise each of the five isolates as follows: An 18 hour statically incubated culture of each *S. zooepidemicus* isolate was diluted 1:4 in PBS, then 6µl of diluted culture was mixed with a separate microcentrifuge tube containing 150µl of one of the rabbit antisera and 450µl of whole, heparinised naive rabbit blood. Control mixtures contained pre-immune rabbit sera, which replaced rabbit antisera; all tubes were mixed at 37°C for 2 hours. At the beginning and end of the incubation period, an aliquot from each tube was serially diluted in PBS and spread plate inoculated onto horse blood agar which was incubated for 18 hours. Blood agar plates with between 30 and 330 colonies were counted and the number of colony forming units per millilitre (cfu/ml) were calculated from the dilution. The percentage reduction in cfu/ml against controls after two hours incubation were calculated. This was repeated three times and the mean percentage growth/reduction calculated.

A 15µl aliquot of an 18 hour statically incubated culture of each *S. zooepidemicus* isolate was mixed separately with each heparinised blood from five 9 month old ponies (from group A, see Chapter 3), for two hours at 37°C. Antibody naturally present acted as an opsonin. The percentage reductions in cfu/ml were calculated using a similar method to that employed for the rabbit antiserum experiments, with the exception that the numbers of bacteria at the start of the incubation was used as a control. Each assay was repeated five times and the mean percentage growth/reduction calculated. Sera from the same ponies were also used in immunoblots against hot acid extracts of each *S. zooepidemicus* isolate as described above.

Ribotyping of isolates with primers for the 16S-23S RNA intergenic spacer.

DNA was extracted from each isolate, then PCR and agarose gel electrophoresis carried out as described by Chanter *et al.* (1996). Briefly, DNA was extracted using guanidine thiocyanate and prepared for PCR using the Wizard DNA Clean Up System. PCR was carried out using a set of eight primers derived from the variant sequences of regions 1 and 5 to 6 of the Lancefield Group C 16S-23S RNA gene intergenic spacer. PCR products were analyzed by agarose gel electrophoresis and each isolate assigned to one of eight types according to which combinations of reactions were positive.

Attempts to develop a murine intranasal challenge model of *S. zooepidemicus* infection

Selection of a susceptible inbred strain of mouse, (Experiment 1). Females of four inbred strains of mouse were tested for susceptibility to intranasal challenge with *Streptococcus zooepidemicus* (C3H, BalbC, C57/BL6 and CBA/CA). Six of each strain were given 5×10^7 cfu of isolate C shaken for four hours in THB intranasally and symptoms noted for five days. After euthanasia, specimens of lung, thymus, myocardium, spleen, kidney and liver were collected in neutral buffered formalin for histopathology. Longitudinal decalcified sections of each mouse head were also prepared to include the nasal and oral cavities, pharyngeal tissues and brain.

Effect of increasing the intranasal challenge dose, (Experiment 2). Six individually marked female CBA/CA mice were given 5.6×10^8 cfu of isolate C shaken for four hours in THB, intranasally. Individual mouse weights were recorded daily and after euthanasia at five days, samples were taken as above for histopathology.

Intranasal challenge and histopathology in mice at post mortem examination at daily intervals, (Experiment 3). Thirty female CBA/CA mice were given 2.1×10^8 cfu of isolate C and six mice were kept as controls. All mice were individually marked and controls were kept separately. Mice were weighed and after daily euthanasia of five test plus one control mouse, histological samples were taken as above with the addition of

pancreas, adrenal gland, thyroid gland, nasal and oro-pharangeal mucosa, brain and mandibular lymph node.

Attempts to enhance the virulence of the *S. zooepidemicus* challenge (Experiment 4).

Isolate C was grown for four hours with 10% sterile foetal calf serum and 4.6×10^8 cfu was inoculated intranasally into each of six female CBA/CA inbred mice. After euthanasia, three days later, specimens of mandibular lymph node, thymus, lung, myocardium, liver, spleen, kidney, pancreas, ovary and uterus were collected in neutral buffered formalin for histopathology.

Development of a murine intraperitoneal challenge model of *S. zooepidemicus* infection

LD₁₀₀ of *S. zooepidemicus* isolates C and D, (Experiment 5). Tenfold serial dilutions in PBS of 18 hour static cultures of isolates C and D in THB were inoculated intraperitoneally into female CBA/CA mice, at doses of between 7 cells and 9.5×10^7 cfu per mouse for isolate C, and between 7 cells and 9.7×10^7 cfu per mouse for isolate D.

Eight mice were inoculated per dilution. After euthanasia, when clinical signs suggested the development of systemic infection, samples of liver, spleen, lung and nares were collected and inoculated onto horse blood agar and incubated for 18 hours.

Vaccination and homologous intraperitoneal challenge, (Experiment 6). Cultures of isolates C and D grown to 1×10^9 cfu/ml were made into bacterins by incubation for four hours with shaking in the presence of 0.01% (v/v) formalin. Each bacterin was separately mixed with an equal volume of FIA and 0.1ml was administered intraperitoneally to separate female CBA/CA mice for each isolate. This was followed by a booster dose, prepared with FIA as the first, one month later. Twenty mice were kept as unvaccinated controls. One month later, as part of what was intended to be immunization to test heterologous challenge, all vaccinates were challenged intraperitoneally with 0.1ml of 18 hour static culture in THB of the homologous isolate and the control mice were similarly challenged for each isolate. Immediately prior to challenge, plasma was collected from

five mice of each group, plus the control group, from the tail tip and allowing approximately 20 μ l of blood to mix with 180 μ l of PBS containing heparin (a dilution of approximately 1:10). After euthanasia, when clinical signs suggested the development of systemic infection, samples of liver, spleen and lung were collected and inoculated onto horse blood agar and incubated for 18 hours. Plasma was collected from mice who recovered from the challenge before euthanasia. All plasma samples were tested against immunoblots of homologous isolate hot acid extracts at a dilution of 1:100 and detected with a horse radish peroxidase labeled anti mouse conjugate (Sigma) at a dilution of 1:2000. All other method details were as above for rabbit antisera immunoblotting.

Results and discussion

Antigenic relatedness of proteins in hot acid extracts of different *S. zooepidemicus* isolates characterized using immune rabbit antisera

SDS-PAGE gels of extracts stained with silver, showed that all of the isolates possessed a number of polypeptides of a similar distribution of molecular masses (Figure 8). Immunoblotting detected antibodies in rabbit antisera to some of the polypeptides seen in stained gels in homologous reactions and fewer in heterologous reactions. For example, twelve polypeptides of isolate A reacted with antibodies in homologous antiserum, but only 6, 11, 6 and 6 polypeptides reacted with antibodies in the antisera to isolates B, C, D and E, respectively (Figure 9-13).

The polypeptides that reacted with antibodies in the different antisera are detailed in Table 6. Some polypeptides of the same molecular mass in all extracts reacted with antibodies in all of the antisera. The 41 KDa polypeptide is a typical example. Other polypeptides present in all extracts, however, did not react with antibodies in all of the antisera. The 52 KDa polypeptide in extract B is an example of this. Examination of the silver stained SDS-PAGE gels showed that this polypeptide was present in extracts of A, C, D and E, but was fainter. Failure of antibodies to react with this in these extracts may have been related to low antigenic weight. Variation in the amounts of some polypeptides in the extracts may have been due to variation in acid hydrolysis between isolates since great care was taken in standardizing the conditions employed for this extraction method. The number and sizes of hydrolysis products may vary for surface proteins on different isolates that are nonetheless antigenically related.

Since antisera to isolates A and C contained antibodies to more polypeptides in the extract of isolate E than antiserum to E, the rabbits immunized with A and C may have responded better, rather than this difference being due to differences in the surface polypeptides of the isolates. However, not all differences may be explained in this way since the antibodies in some homologous antisera reacted with more polypeptides in homologous extracts than any of the heterologous extracts, suggesting real antigenic differences. The antiserum to

isolate B was a clear example of this (Table 5). Nonetheless, the immunogenic and antigenic hydrolytic breakdown products were similar in molecular mass between isolates and antibodies in rabbit antisera suggest that they are strongly antigenically related.

Antigenic relatedness of polypeptides in hot acid extract proteins of *S. zooepidemicus* isolates characterized using equine sera

Three separate sets of pony sera designated A, B and C, were tested for antibody to polypeptides in hot acid extracts of each isolate separated by SDS-PAGE and transferred to nitrocellulose.

All twenty-one of the group A ponies had antibodies to a 52KDa polypeptide in extracts of isolates A and D, whilst few had antibody to the 52KDa polypeptide in extracts of isolates B and E, and none to an equivalent polypeptide in the extract of isolate C. This was in spite of a possible problem of insufficient antigen in extracts of A and D suggested by the results obtained with the rabbit antisera. Since rabbit antisera to four of the isolates contained antibody which reacted with this polypeptide in the extract of B, the lack of antibody in equine sera which nonetheless reacted with a 52 KDa component in some of the other isolates suggest antigenic variation. Furthermore, this difference could only be identified using antibodies present naturally in equine sera.

Thirteen ponies had antibody to polypeptides of 46 to 36KDa in the extract of isolate D, but antibodies reacting with this range of polypeptides in the extracts of the other isolates were present in far fewer ponies. Sixteen, thirteen, eight and zero ponies had antibody to a 46KDa polypeptide in extracts of isolates B, C, A and E respectively. Nineteen ponies had antibody to the 35KDa polypeptide in extracts of isolates B and C, which was not reactive with the 36KDa polypeptides in extracts of the isolates A or E.

Group B pony sera (n=12) had no antibody to the polypeptides in the extract of isolate C, but there was antibody to a range of polypeptides in the extracts of isolates A and B and at

least the 46 and 35KDa polypeptides in the extracts of isolates D, and E. Ten ponies had antibody to a 52KDa polypeptide in the extract of isolate A.

Group C pony sera (n=8), did not have any antibody to the polypeptides in the extracts of the isolates C and D, but had antibody to a broad range of polypeptides in the extract of isolate B. There was a narrower range of antibody reactivity for polypeptides in the extracts of the isolates A and E.

The sera of all three groups of ponies were more discriminating between the antigens of different isolates than the rabbit antisera and showed less cross-reactivity.

Characterization of the *S. zooepidemicus* isolates using rabbit opsonic antisera

The opsonic activity of antisera to extracts of the five isolates were tested against the homologous and heterologous isolates (Table 7). Heparinised non-immune rabbit blood was used as a source of neutrophils rather than equine cells to rule out the possible lack of recognition of rabbit opsonins by equine polymorphonuclear leukocytes. Sera from the rabbit blood used as a source of neutrophils and complement for the assay did not show any reactivity for polypeptides in any of the extracts of isolates A-E in immunoblots of SDS-PAGE gels and pre-immune serum was not opsonic in blood from these animals. This was a surprising result because antisera to isolates A and E were opsonic for other isolates.

Isolates B and C were uniformly phagocytosed by all five antisera. The behavior of isolate D was more complex, with a reduction in cfu's for two replicates of the assay, but growth in another, even with the homologous antiserum. Isolate A was generally not phagocytosed. Isolate E grew in the presence of all antisera including the homologous antiserum.

Characterization of the *S. zooepidemicus* isolates using equine whole blood

Equine blood was used as a natural source of antibody as well as complement and polymorphonuclear leukocytes, which might have the potential to discriminate between opsonogenic antigens of the different isolates according to their history of related *S. zooepidemicus* infection (Table 8). Five ponies from group A were bled on five occasions over a ten day period. Isolates B and C were phagocytosed by all of the blood samples. Isolate A was resistant to phagocytosis in the first two blood samples but susceptible in subsequent samples suggesting the development of immunity over the period during which blood samples were taken; some nasal mucopurulent discharge and coughing associated with the presence of *S. zooepidemicus* was noticed in this group of animals.

Isolates D and E were inconsistently phagocytosed and, therefore, results for these were difficult to interpret. Blood from two of the ponies reduced cfu's of isolate D but not on all occasions. Isolate E was generally susceptible to phagocytosis with the blood of all of the ponies but not always to as great an extent as seen with isolates B and C.

Reaction of antibodies in sera from ponies used as a source of blood for phagocytosis assessed against extracts of the *S. zooepidemicus* isolates in immunoblots of SDS-PAGE gels

Sera from all ponies collected at the end of phagocytosis tests (Table 9), reacted strongly with the 41 and 46KDa proteins in the extract of isolate A, but only weakly with the 72 KDa polypeptide. The blood of two ponies (1 and 2) which reduced the numbers of isolate A the most in phagocytosis assays, reacted most strongly with the 46KDa polypeptide. Otherwise, there were a cluster of faint bands in the extract of isolate A ranging from 27-48KDa, to which all five ponies had antibodies and which did not appear related to opsonisation.

Isolate B was uniformly phagocytosed by blood from all of the ponies and surprisingly in contrast to earlier samples, their sera reacted strongly with a 52KDa polypeptide in the extract of isolate B. The first serum sample characterized by immunoblotting (see above),

which lacked antibody to this polypeptide was taken six months earlier. A cluster of polypeptides of isolate B, between 26.5 and 48KDa, reacted with antibodies in sera of all of the ponies but to different extents.

Isolate C was generally phagocytosed by all pony bloods but not to the same extent as isolate B. Antibodies in all sera reacted with polypeptides between 50 and 29KDa in the extract of isolate C. The 36 and 44KDa polypeptides were the most strongly detected of these. Polypeptides of 72, 63.5, 59, and 54KDa were also detected by antibodies in all of the sera but with less intensity.

Four of the ponies did not have antibodies to the polypeptides of isolate D, which was barely phagocytosed at all; pony 3 had barely detectable antibodies to polypeptides of 84, 78.5, 52 and 41KDa, which did not seem to confer opsonic ability. All sera contained antibodies to the polypeptides of isolate E of 33 to 21KDa and blood from these ponies generally phagocytosed this isolate. In contrast, the rabbit antisera were not opsonic and did not react with the 33 KDa polypeptide. It is more difficult to determine whether the 21 and 22 KDa polypeptides detected by the different species of antisera were distinct or not.

Ribotyping of isolates with primers for the 16S-23S RNA intergenic spacer

Isolates A, C and D were type A1, which had regions 1a,5a,6a and 7a. Isolate E was type D2, which had regions 1b,3,5b,6b and 7c. Isolate B was type B1, which had region 1a (like type A1) and regions 5b,6b and 7c (like type D2). To assess if there was any association between ribotype and the possession of other properties, all analyses of *S. zooepidemicus* variation were examined for factors which might distinguish isolates E and B, but unite the others. In immunoblots of hot acid extracts with rabbit antisera (25KDa polypeptides and above), it was found that there was antibody detectable to a broader polypeptide size range for isolate B than isolates A,C and D, and antibody to a narrower range in isolate E, although there was no correlation between ribotype and antibody raised to specific polypeptides.

Attempts to develop a murine intranasal challenge model of *S. zooepidemicus* infection

Selection of a susceptible inbred strain of mouse, (Experiment 1). Four of the CBA/CA mice challenged with 5×10^7 cfu of isolate C had significant histological lesions, whereas only one of the C57/BL6 mice and none of the BalbC or C3H mice were affected. CBA/CA mice were selected for further development of the challenge model. Of the four CBA/CA affected mice, two had spleen, heart and lung lesions, one had meningitis and early splenitis, and one had pharyngeal abscessation and cellulitis.

Effect of increasing the intranasal challenge dose, (Experiment 2). To increase the proportion of affected mice the challenge dose was increased to 5.6×10^8 cfu of isolate C. However, three days after challenge, there were no gross lesions visible on dissection or inflammation present, but although surface colonization of lingual and oropharyngeal mucosa by small to moderate numbers of streptococcus-like bacteria were seen in all mice. One of the mice developed abscessation of the sub-mandibular lymph nodes.

Intranasal challenge and histopathology in mice at post mortem examination at daily intervals, (Experiment 3). All of the test mice on day three had lesions; These were in the spleen and mandibular lymph nodes, but lesions from mice examined post mortem on other days were sporadic. The relative lack of lesions in mice examined more than three days after challenge was regarded as an indication of the inconsistency of the model. There should have been lesions present in mice examined four days after challenge if at three days they had lesions of the severity seen in the group that was examined three days after challenge.

Attempts to enhance the virulence of the *S. zooepidemicus* challenge, (Experiment 4). Experience with *S. equi* has shown from young cultures that serum may enhance the virulence of the inoculum. This approach was taken with *S. zooepidemicus*. Only one mouse of the six, challenged with 4.6×10^8 cfu grown with serum, had lesions, which were in the mandibular lymph nodes. The intranasal route of challenge was too inconsistent on which to base a model to demonstrate the effects of vaccination on.

Development of a murine intraperitoneal challenge model of *S. zooepidemicus* infection

LD₁₀₀ of *Streptococcus zooepidemicus* isolates C and D, (Experiment 5). The LD₁₀₀ for isolate C was 9.6×10^6 cfu/ml and for isolate D was 9.6×10^8 cfu/ml (Figure 14). *S. zooepidemicus* was isolated from the spleen, liver and lung of all mice after euthanasia, but none were isolated from the nares.

Vaccination and homologous intraperitoneal challenge, (Experiment 6).

S. zooepidemicus was isolated from samples of spleen, liver and lung from all mice after euthanasia as soon as clinical signs suggested a terminal septicaemia. Vaccination did not prevent septicaemia, but did delay the onset of symptoms. Vaccinates homologously challenged with isolate C survived for 24 hours or more and one recovered completely, whilst control mice challenged with isolate C survived for less than 24 hours. Similarly, vaccinates homologously challenged with isolate D survived for 18 hours or more, whilst control mice challenged with isolate D survived for less than 18 hours.

Only one mouse survived the experiment, in the group vaccinated and challenged with isolate C. Murine plasma collected immediately prior to challenge had poor reactivity to the hot acid extract of isolate C, but the surviving mouse had a strong reaction to a range of polypeptides from approximately 25 to 52 KDa and also to several low molecular mass polypeptides.

Table 6. Polypeptides (approximate molecular mass in KDa) in hot acid extracts of *S. zooepidemicus* isolates A,B,C,D and E, which react with homologous and heterologous antisera.

Extract	Antiserum				
	A	B	C	D	E
A	9a 9b 9	9a 9b 9	9a 9a2 9b 9	9a 9b 9c	9a 9b 9
	11 12 17 18	17	11 14 18	17	17
	20 23 25	25	22 23 25	25	20
	40 41	41	41	41	41
B	9 (trace)	9	9 (trace)		9
	11 14 15	11 14 15 17	14		11 15
		25 29			
		32 34			
C	52 60	52 60	52		52
	9a 9b 9	9a 9b 9	9a 9a2 9b 9	9a 9b 9c	9a 9b 9
	11 17 18	18	11 18	17	
	22	23	23		
	35	35	35	35	35
D	46	46	46	46	46
	9b 9c 9	9b 9c 9	9a 9a2 9b 9c 9	9b 9c 9	9a 9b 9
			11		
	25	25	25	25	25
E	41	41	41	41	41
	9a 9b 9	9a 9b 9	9a 9a2 9b 9	9a 9b 9c 9	9a 9b 9
				17	
	23	22 23	22 23		
		35	35		
		25	25		25

Table 7. Percentage reduction of colony forming units of each *S. zooepidemicus* isolate in rabbit blood containing homologous and heterologous rabbit antisera after 2 hours incubation.

Isolate	Rabbit antiserum				
	A	B	C	D	E
A	26.5	10	-30.6	-18.2	19.1
	51.8	60.2	59.8	63.4	71.4
	40.5	59.9	48.3	74.6	61
Mean	39.6	43.4	25.8	39.8	50.5
B	-96.4	-96.3	-90.9	-96.3	-92.1
	-83.6	-87.4	-85.7	-93.2	-83.1
	-96.1	-74.7	-95.7	-96.5	-95.8
Mean	-92	-86.1	-90.8	-95.3	-90.3
C	-96.4	-97	-97	-94.4	-95.2
	-87.2	-85.8	-86.5	-51.1	-92.3
	-85.4	-73	-91	-91.7	-91.4
Mean	-89.7	-85.3	-91.5	-79.1	-93
D	-1.7	-68.1	-56.9	-52.6	-62.9
	58.4	>71.2	>71.2	41.6	22.1
	-70.6	-40.4	-64.2	-60.6	-62.1
Mean	-4.6	-12.4	-16.6	-23.9	-34.3
E	82.3	92.4	90.4	81.7	83.3
	85.4	87.1	>88.5	>88.5	>88.5
	>30	>30	>30	>30	>30
Mean	65.9	69.8	69.6	66.7	67.3

Positive values indicate growth.

Table 8. Percentage reduction of colony forming units in two hours, of each *S. zooepidemicus* isolate in the blood of five young ponies after 2 hours incubation; experiment repeated on five separate occasions.

Isolate	Pony number				
	1	2	3	4	5
A	53.6	28.8	0	45.2	63.2
	84.6	71.6	87.9	91.6	97.8
	-20.8	-65.3	66.4	71.6	35.7
	0	-70.6	-13.8	-55.9	-80.3
	-60.6	-40.1	-8.2	6.3	-30.5
Mean	11.4	-15.2	26.5	31.8	17.2
B	-56.1	-74.7	-77.3	-46.4	-75
	-24.7	-69.5	67.7	60.9	-50.5
	-83.7	-63.7	-21.6	-56.9	-100
	-6.7	-8	-61.7	-36.7	-77.5
	-89.5	-45.8	-36.9	-87.8	-80.6
Mean	-52.1	-52.3	-26	33.4	-76.7
C	-48	-80.3	-84.4	-2	-19.8
	59.1	-18.1	65.2	54.2	-44.5
	-70.4	-89.4	-17.3	-19.9	-87.5
	-36.4	17.4	-47.6	-61.8	-71.9
Mean	-23.9	-42.6	-21	-7.4	-55.9
D	42.3	-61.4	28.5	81.2	82.1
	78.6	-10.4	80.2	66.6	63.5
	23.2	-23.3	34.9	46.7	-80.5
	28.1	24.6	39.5	18.6	8.5
	24.4	56.3	25.2	29.7	-44.9
Mean	39.3	-2.8	41.7	48.6	-20.7
E	-87.5		-89.6	-23.8	21.2
	63.7	-62.6	>72.2	>72.2	68.8
	-87.3	-84.8	-8.1	-28.1	-85.1
	-14.7	-32.2	10	-2.1	-64.7
	-7.4	-27.5	-8.8	-12	-43.5
Mean	-26.2	-51.8	-4.9	1.2	-20.6

Positive values indicate growth.

Table 9. Polypeptides (approximate molecular mass in KDa) in hot acid extracts of *S. zooepidemicus* isolates A,B,C,D and E, which reacted with antibody in the sera of the ponies in group A used in phagocytosis assays (sera collected at the time of the fifth experiment).

Isolate	Pony sera				
	1	2	3	4	5
A	82 72 63	82	82	82	82
	48 46 41	48 46 41	48 46	48 46 41	48 46 41
	38 34 33 30	38 34 33 30	33 30	33 30	33 30
					27
B	52	52	52	52	52
	48 46 41	48 46 41	48 46 41		48 46 44 41
	32	34 32	34 32	34 32	39 37 36 34 32
	29 26		29 26		29 26
C	72 63 59 54	59 54	72 63 59 54	72 63 59 54	72 63 59
	*	*	*	*	*
	33 32	33 32	33 32	33 32	33 32
	29				
D			84 52		
			41		
E	32	33 32	33 32	33	33
	21	21	29 21	22 21	29 22 21

* = a series of fine bands on immunoblots ranging from 44 to 36 KDa.

Figure 8. Silver stained SDS-PAGE of *S. zooepidemicus* polypeptides (approximate Molecular Mass in KDa) in hot acid extracts from isolates A, B, C, D and E. Lanes 1-5 show extracts of isolates A-E respectively.

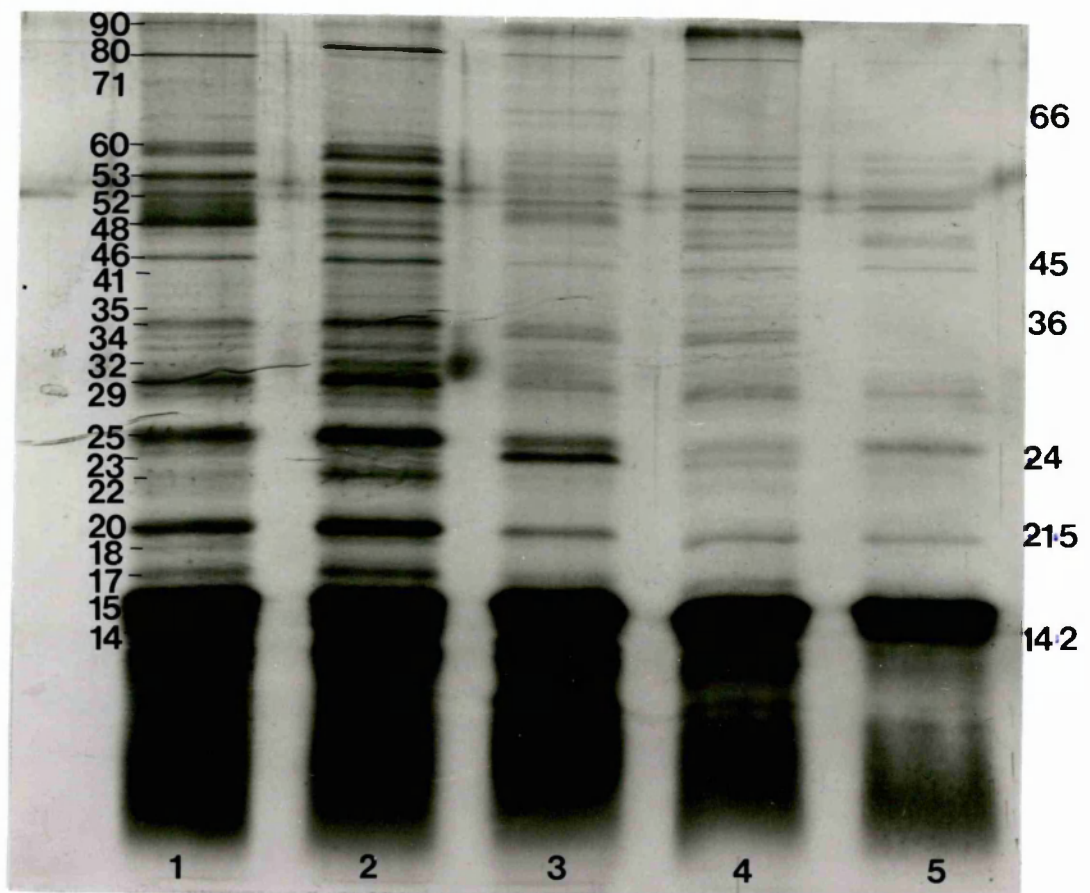


Figure 9. Immunoblot showing the reaction of rabbit antisera to hot acid extracts of *S. zooepidemicus* isolates A, B, C, D and E with isolate A polypeptides (approximate Molecular Mass in KDa). Lanes 1-5 show antisera to extracts of isolates A-E respectively.

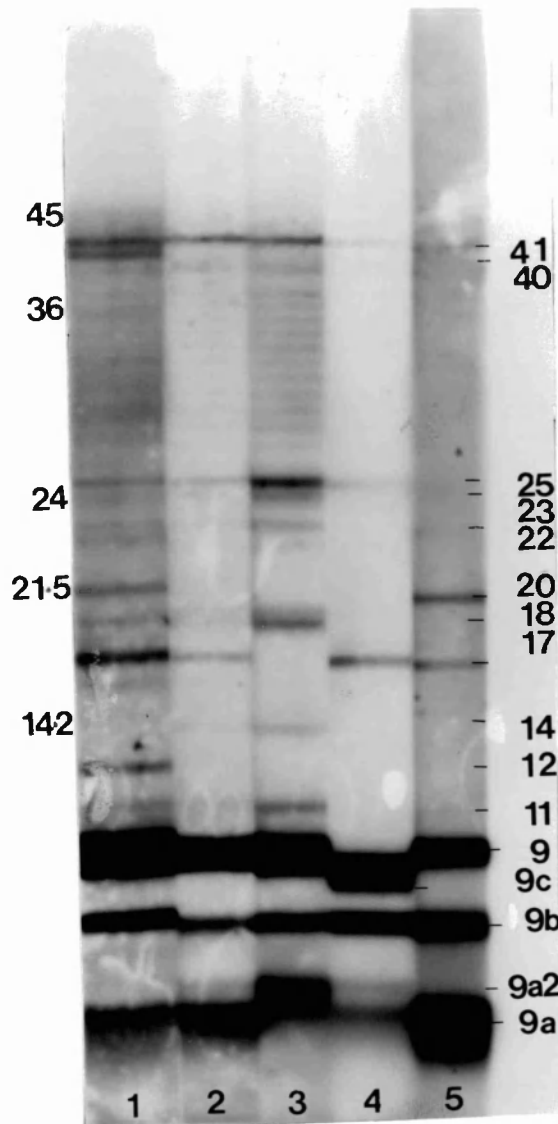


Figure 10. Immunoblot showing the reaction of rabbit antisera to hot acid extracts of *S. zooepidemicus* isolates A, B, C, D and E with isolate B polypeptides (approximate Molecular Mass in KDa). Lane 1 shows antisera to extract of isolate E, lane 2 to D, lane 3 to B, lane 4 to C and lane 5 to A.

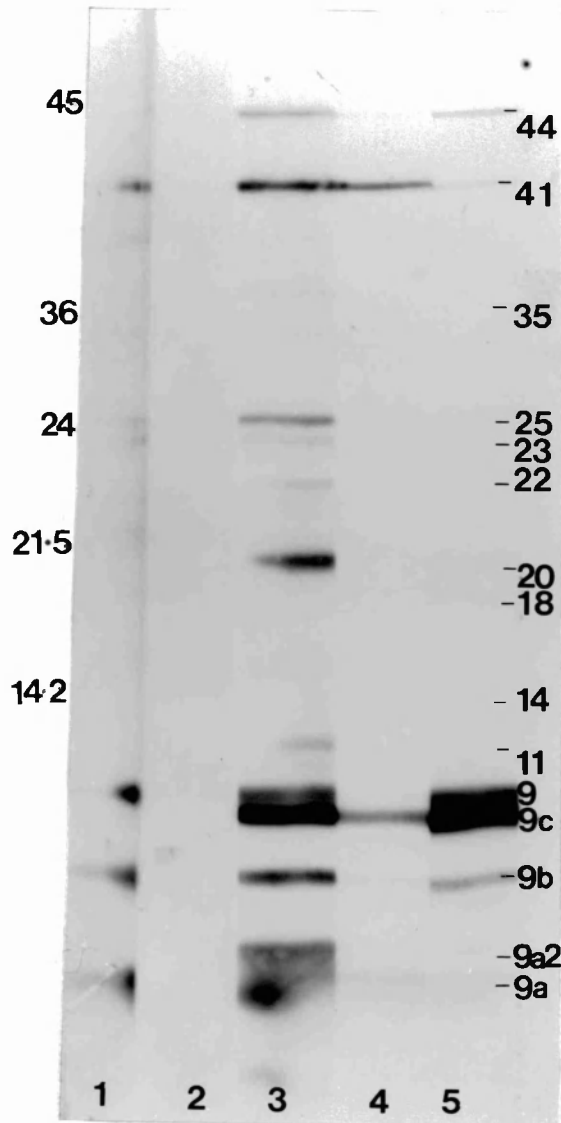


Figure 11. Immunoblot showing the reaction of rabbit antisera to hot acid extracts of *S. zooepidemicus* isolates A, B, C, D and E with isolate C polypeptides (approximate Molecular Mass in KDa). Lanes 1-5 show antisera to extracts of isolates A-E respectively.

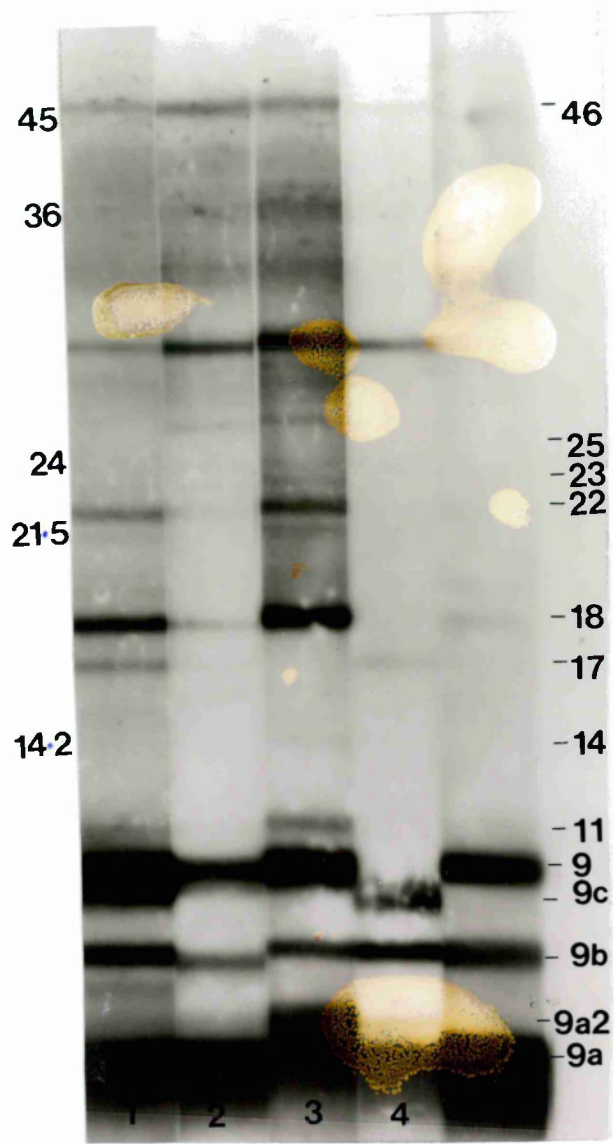


Figure 12. Immunoblot showing the reaction of rabbit antisera to hot acid extracts of *S. zooepidemicus* isolates A, B, C, D and E with isolate D polypeptides (approximate Molecular Mass in KDa). Lanes 1-5 show antisera to extracts of isolates A-E respectively.

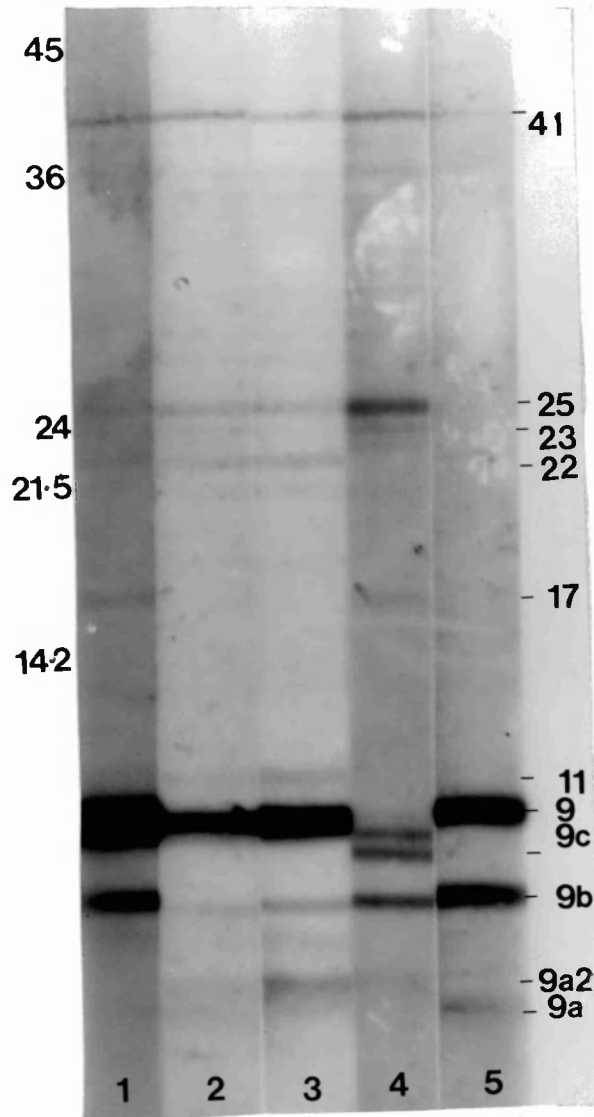


Figure 13. Immunoblot showing the reaction of rabbit antisera to hot acid extracts of *S. zooepidemicus* isolates A, B, C, D and E with isolate E polypeptides (approximate Molecular Mass in KDa). Lanes 1-5 show antisera to extracts of isolates A-E respectively.

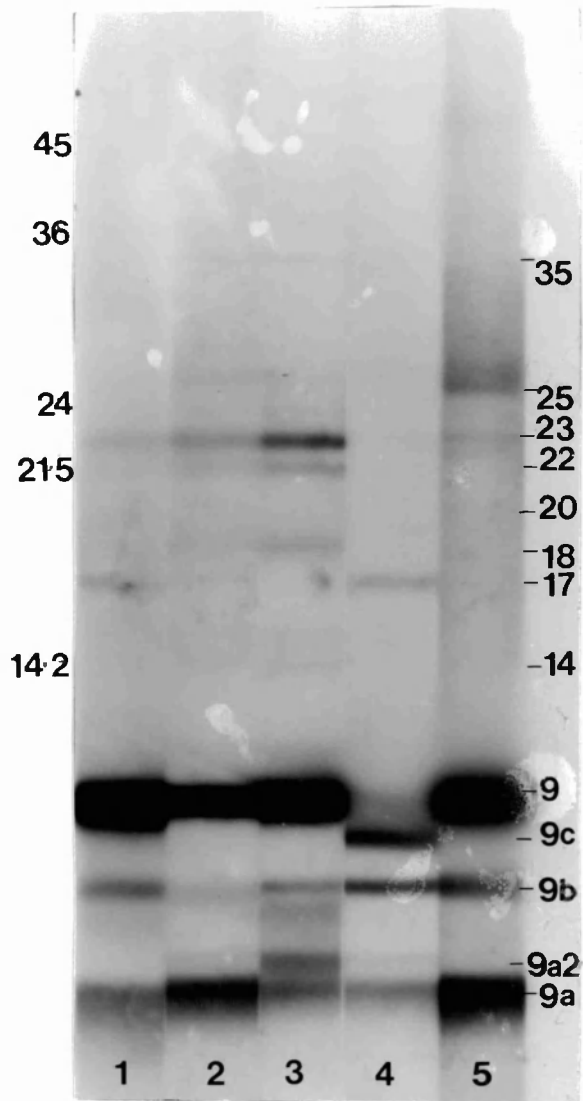
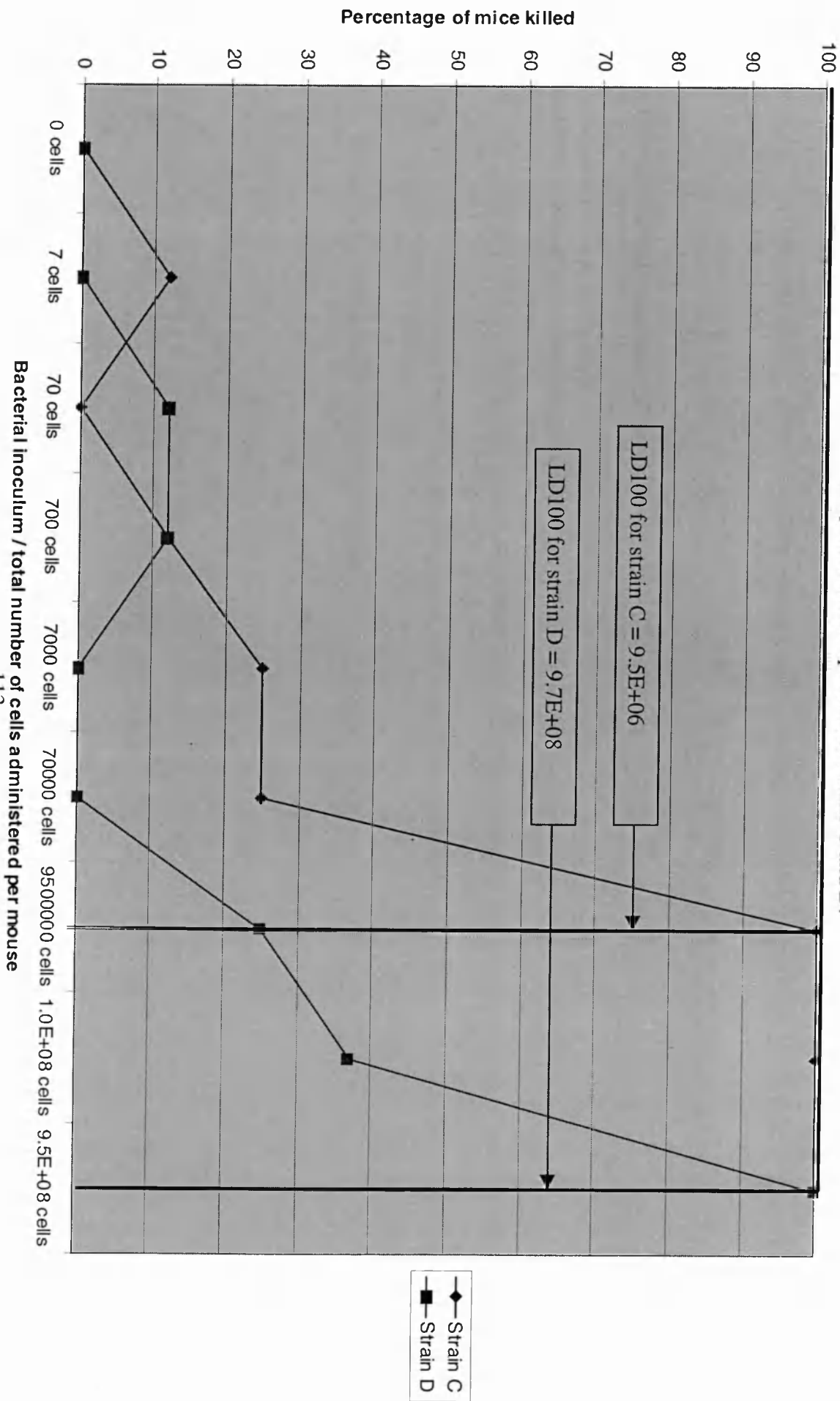


Figure 14. Percentage of mice killed in *extremis* after intraperitoneal inoculation with different numbers of *Streptococcus zooepidemicus* strains C and D.



5.0 CHAPTER FIVE

CHARACTERISATION AND CLONING OF A STREPTOCOCCAL HYALURONATE ASSOCIATED PROTEIN AND ITS ASSESSMENT AS A PROTECTIVE IMMUNOGEN IN A MURINE *STREPTOCOCCUS* *ZOOEPIDEMICUS* CHALLENGE MODEL.

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Objectives

The aims of this work were: i) to attempt to purify the equivalent of the *S. equisimilis* HAP from a highly encapsulated strain of *S. equi*, from culture supernatant, using a modified cholate-solubilization method (Prehm and Mausolf, 1986); ii) to test for the presence of HAP by PCR using primers based on the DNA sequence previously published for *S. equisimilis* (Lansing *et al.* 1993); iii) to clone and express the *S. equi* HAP, if present; iv) to produce antibodies to HAP and recombinant to assess their effects on capsulation *in vitro*; and v) to assess the possibility of protecting against *S. zooepidemicus* in a mouse model of infection.

Summary

The capsule of *S. equi*, the cause of strangles, and *S. zooepidemicus*, associated with equine lower airway disease, plays an important role in evading phagocytosis by polymorphonuclear leucocytes. It is composed of hyaluronate, making it non-immunogenic. A protein, previously described as being strongly bound to cell associated and dissolved hyaluronate (hyaluronate associated protein or HAP) from *S. equisimilis*, was investigated (a) for its presence in *S. equi* and *S. zooepidemicus* and (b) as a surrogate immunogen for the capsule structure with the potential to protect against experimental challenge of mice. The purified capsule of *S. equi* contained a protein of similar molecular mass to the *S. equisimilis* protein (approximately 53 KDa). Primers derived from the published sequence of the *S. equisimilis* protein in a polymerase chain reaction (PCR) yielded a product from *S. equi* and *S. zooepidemicus* of the expected size and endonuclease restriction fragment pattern. The PCR product from *S. equi* was blunt end cloned in frame into the expression vector pP_L-Lambda. However, when expression was induced in *E. coli* N4830-1 the recombinant plasmid was stable only if cloned in the orientation opposite to that required for expression. Subcloning of two large in frame Ssp I fragments of the HAP gene, approximately equivalent to the two halves of the molecule, into the expression vector pGEX-3X yielded only the back half in the correct orientation (Fragment 4). Two overlapping fragments of the front half would not express in pGEX-3X (Fragments a and b), so the front half was expressed as three non-overlapping fragments (Fragments 1,2 and 3). Rabbit antiserum to the native protein in purified hyaluronate reacted strongly in immunoblots with the glutathione S transferase (GST) fusion product. This antiserum and antiserum to the recombinant fusion protein, when soaked into filter paper strips, caused a diminution of capsule production by *S. equi* cultured on blood agar. Neither antiserum supplemented into fresh rabbit blood was opsonic for *S. equi*. Immunization with recombinant HAP significantly increased survival time and clearance of bacteria in mice challenged intraperitoneally with *S. zooepidemicus*.

Materials and methods

Bacteria and culture media

An isolate of *S. equi* from the sub-mandibular abscess of a New Forest pony with Strangles, in 1990, and *S. zooepidemicus* isolate C (see Chapter 4), were used throughout; both were stored at -50C on cryobeads (Microbank). Stored cultures were revived and maintained by culture on horse blood agar (Wilkins-Chalgren anaerobe agar (Unipath Ltd), supplemented with 5% (v/v) citrated horse blood) at 37C for 18 hours; where appropriate sub-cultures were made in Todd-Hewitt broth at 37C for 18 hours (THB; Unipath). Challenges for mice were made by using a 5% (v/v) inoculum of an overnight THB culture into THB and incubation at 37C with shaking at 200 rpm for 4 hours.

The transformation competent *E. coli* strains N4830, N99, JM109, TG1 and DH10B used for recombinant HAP production were stored at -70C in 20% (v/v) glycerol in Luria Bertani broth (LB); these were propagated in LB broth, or LB agar, with added 80µg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), 100µM isopropyl-thiogalactoside (IPTG) and 100µg/ml ampicillin (Sigma) when appropriate. All incubations of THB and LB cultures were at 37C unless stated otherwise and blood agar plates were incubated at 37C in the presence of 5% (v/v) carbon dioxide in air.

SDS- polyacrylamide gel electrophoresis

For purified HAP and recombinant HAP expression studies, 10µl volumes of each clone, or column fraction and molecular mass markers (Sigma), boiled for 5 minutes in Laemmli sample buffer containing marker dye, were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), as previously described in Chapter 3, with a 12% (w/v) polyacrylamide separating gel and a 4% (w/v) stacking gel in a Mini Protean II apparatus (BioRad) and a constant voltage of 200V at room temperature for approximately 45 minutes. Proteins in the gels were detected by silver staining, as described in Chapter 3.

Isolation of native HAP using cholate solubilization methods

Isolation and purification methods described previously (Prehm and Mausolf, 1986), with modifications, were applied to the culture supernatant of a highly encapsulated *S. equi* culture, pre-diluted to 20% (w/v) culture in 150mM phosphate buffered saline, pH 7.2 (PBS). Chemicals were supplied by Sigma.

Briefly, 1500 ml of diluted culture prepared as above, was centrifuged for 40 minutes at 9000g and 4°C. The supernatant and an aqueous solution of 10% (w/v) cetylpyridinium chloride was prewarmed to 37°C; the two were mixed to a final concentration of 1% (w/v) cetylpyridinium chloride and stirred at 37°C for 30 minutes, then centrifuged at 10 000g at 4°C for 5 minutes. The pellet was suspended in 40 ml of 10% (w/v) sodium acetate in methanol, then centrifuged at 10000g for 3 minutes. This pellet was resuspended in 1 ml of 40mM aqueous sodium phosphate buffer, pH 6.8, containing 1% (w/v) sodium cholate. The solution was prepared for application to a 1 ml Hydrophobic Interaction Chromatography column (HIC, Pharmacia), by diluting 1:4 in 40mM sodium phosphate buffer containing 1% (w/v) sodium cholate and solid ammonium sulphate was dissolved in it to a final concentration of 1M. The column was equilibrated at room temperature with 5ml of 40mM sodium phosphate buffer (pH6.8) containing 1% (w/v) sodium cholate, followed by 5ml of 40mM sodium phosphate buffer (pH6.8), with 1M ammonium sulphate containing 1% (w/v) sodium cholate. A 2ml volume of prepared sample was applied and the flow through was collected as two 1ml fractions. The column was washed with 5ml of 40mM sodium phosphate buffer (pH6.8), with 1M ammonium sulphate containing 1% (w/v) sodium cholate and 1ml fractions were collected. Aliquots of 2ml 40mM sodium phosphate buffer (pH6.8), with 0.8, 0.6, 0.4, 0.2M, or no ammonium sulphate containing 1% (w/v) sodium cholate, were applied to the column sequentially. Fractions (1ml) were collected, then the column was washed with 5ml water and further fractions collected. All fractions collected were examined by silver stained SDS-PAGE gels (see above). The protein concentrations of fractions identified as likely to contain HAP were measured using a bicinchoninic acid assay (Pierce).

Amplification, cloning and expression of recombinant HAP

Preparation of template DNA for PCR

Streptococcal genomic DNA was extracted using an alkaline lysis method similar to the one used by R. Treisman and as described by Sambrook *et al.* (1989). A washed bacterial pellet from a 500ml culture (obtained as above) was resuspended in 18ml of solution I, comprising 50mM glucose, 25mM Tris HCl (pH 8.0) and 10mM EDTA (pH 8.0). A freshly prepared solution of lysozyme (2ml of 10mg/ml in 10mM Tris HCl, pH 8.0) was added, followed by 40ml of solution II, comprising 0.2N NaOH and 1% (w/v) SDS; the tube was mixed by several inversions, then incubated at room temperature for 10 minutes. Ice cold solution III (20ml), comprising 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml of water, was mixed in and the tube incubated on ice for 10 minutes. The tube was then centrifuged at 10000 g and 4C for 15 minutes and the supernatant fraction re-centrifuged as before. The supernatant fraction was transferred to a fresh tube and 0.6 volumes of isopropanol added. After thorough mixing, the tube was centrifuged at 16C and 12000 g for 5 minutes, and the supernatant fraction carefully decanted off, leaving a pellet that was rinsed with 70% (v/v) ethanol. The pellet was dried at 37C for 30 minutes, then dissolved in 3ml of TE buffer (pH 8.0).

PCR and cloning of HAP in the expression vector pPL-Lambda

PCR of HAP DNA:

A forward primer: 5'AAGGCCTATGACAGTACTAGGAACAAA-3' (base position 1-20 on the published sequence) and reverse primer:

5'- AAGGCCTACGGAAGGTAAAGGGGAT-3' (base position 3' (base position 1550-1569 on the published sequence), to amplify the whole HAP open reading frame, was designed from the published *S. equisimilis* sequence (Lansing *et al.* 1993). Primers were used at 3.5µl of 50µg/ml reaction, to amplify the HAP sequence with flanking *Stu*I restriction enzyme sites, by polymerase chain reaction (PCR) (Mullis *et al.* 1986). *S. equi* genomic DNA was used as a template for PCR; 0.5µl (10ng) of DNA was added to a 50µl PCR reaction volume. PCR buffer (5µl; 100mM Tris HCl, pH 8.3, 15mM MgCl₂,

500mM KCl, 1mg/ml gelatin; Boehringer), dNTPs (8 μ l of a 1.5mM equal mixture; Perkin Elmer) and 29 μ l of water were added before overlaying the reaction with mineral oil (Sigma). Cycling conditions were 95C for 5 minutes during which 0.5 μ l of Amplitaq DNA polymerase (Perkin Elmer) was added, followed by 30 cycles of 94C for 1 minute, 55C for 1 minute and 72C for 3 minutes. A negative control PCR reaction was set up in the same way, replacing the template DNA with water to monitor contamination.

Analysis of HAP PCR products: The quality, size and quantity of the PCR product was checked by first mixing 2 μ l of product with an equal volume of EDTA sample buffer containing bromothymol blue dye, then loading it onto a 1% (w/v) agarose gel set in gel apparatus (Pharmacia), along with 2 μ l each of 1Kb and Lambda DNA molecular weight marker (Sigma) loaded into an adjacent lane. The gel was run at 60 Volts for 60 minutes, then stained for 30 minutes with 0.05% (w/v) ethidium bromide in tris-acetate EDTA buffer containing 0.04M tris-acetate and 0.001M EDTA. The gel was viewed on an UV transilluminator and photographed using a thermal image camera (Sony).

The quality of the PCR product was assessed visually; acceptable quality was deemed as the presence of only one clean band of the correct size in the test and positive control lanes, and nothing in the negative control lane. The size of the PCR product was estimated from the photograph by firstly plotting the log of the marker molecular weights in Kb, against the distance migrated from the wells in millimetres, then measuring the distance migrated for the PCR product. A log value for the PCR product was read from the graph and the inverse taken to obtain the molecular weight. An estimate of the concentration of PCR product or plasmid was estimated by comparing the intensity and width of a band with Lambda DNA molecular weight marker bands, which were of known DNA concentration.

To check for the presence of the gene in *S. zooepidemicus*, PCR was also conducted using the above primers and conditions, except that similarly extracted *S. zooepidemicus*

genomic DNA was used as a template. The PCR product (concentration estimated to be 1µg) was cleaned and digested with 2units of Pst I (Pharmacia). One unit of restriction enzyme was defined by the manufacturer as the amount needed to completely digest 1µg of lambda DNA to completion in 60 minutes (usually 37C), in a total assay mixture of 50µl. All restriction enzymes used were supplied by Pharmacia. 10x One-Phor-All Plus (OPA+) buffer (Pharmacia), comprising 100mM Tris-acetate, pH7.5, 100mM magnesium acetate and 500mM potassium acetate, was added to a final concentration of 1X as recommended by the manufacturer for this enzyme, and the volume was made up to 50µl with water. A concentration of 1X OPA+ was used throughout unless specified otherwise. The PCR product was digested for 18 hours at 37C, then the restriction enzyme was inactivated by incubating the mixture at 85C for 15 minutes. The resulting fragment sizes, determined by agarose gel electrophoresis in the same way as described below, were compared with the predicted sizes calculated by counting the base pairs in the predicted sequence.

Restriction enzyme digestion of HAP PCR products: The PCR product generated as above, was cleaned using a Wizard DNA clean up kit (Promega), a size exclusion mini-column based kit designed to remove Taq Polymerase enzyme and buffer ions which might inhibit digestion with restriction enzymes. The kit was used according to manufacturer's instructions, except that the DNA was eluted in 30µl of water at 85C, rather than 50µl at 65C. The entire cleaned PCR product was digested with 2 units of Stu I. 10x OPA+ buffer was added to a final concentration of 2X (10µl) as recommended by the manufacturer for this enzyme, and the volume was made up to 50µl with water. The PCR product was digested for 18 hours at 37C, then the restriction enzyme was inactivated by incubating the mixture at 85C for 15 minutes.

Digestion and dephosphorylation of and pPL-Lambda cloning vector: The expression vector pP_L-Lambda was developed for cloning products toxic for *E. coli* by employing tight temperature sensitive repression of the P_L promoter (Pharmacia). The vector was initially digested with 5 units of Hpa I as described above, and then dephosphorylated to prevent

the vector ligating to itself. This was done by mixing 0.2 units (0.2 μ l) of calf alkaline phosphatase (Pharmacia) with 10 μ l of the digested vector. (OPA+ was already present in the recommended concentration because it was added for the restriction enzyme digestion step). The mixture was incubated at 37C for 30 minutes and the alkaline phosphatase enzyme was inactivated by incubating at 85C for 15 minutes. The concentration of digested, dephosphorylated vector was determined as described for PCR products, above.

Ligation of HAP PCR product and pPL-Lambda vector: The amount of PCR product (insert) required to mix with 20-50ng of pPL-Lambda vector was calculated by dividing the size of the vector in Kb, by the size of the insert in Kb, then multiplying by the amount of vector DNA and the molar ratio of insert: vector recommended by the manufacturer (Pharmacia). T4 DNA ligase (Pharmacia) was added in excess such that 2 units were added to a 10 μ l reaction volume. One unit of ligase enzyme was defined by the manufacturer as the amount needed to catalyze the formation of 1nmol of phosphatase-resistant [³²P]Pi from 10 μ M 5'-[³²P]-oligo (rA)₁₁ in 30 minutes at 37C. A final concentration of 1X OPA+ and 1mM ATP were added and the volume was made up to 10 μ l with water. A negative control ligation was set up in the same way, replacing the insert with water, to check both for contaminating DNA and to ensure that the vector is not re-ligating back to itself. A positive control ligation was also set up using the positive control insert DNA supplied with the vector, to test that the reagents were not defective. The mixtures were incubated for 18 hours at room temperature, then the T4 DNA ligase was inactivated by incubation at 65C for 15 minutes.

Transformation of competent cells with HAP ligated into pPL-Lambda: The N4830 and N99 cells were not supplied as competent for transformation, so both required preparation as follows. One colony picked from an LB agar plate was used to seed a 4ml LB broth grown statically for 18 hours at 4C. This culture was then used to seed a 100ml LB broth incubated at 37C with 225rpm shaking, for 2 hours. The resulting culture was centrifuged at 10000 g and 4C for 5 minutes. The pellet was resuspended in 0.1M calcium chloride, incubated on ice for 15 minutes and then centrifuged as before. The pellet was

resuspended in 4ml of 0.1M calcium chloride and incubated on ice for 4 hours. The competent cells were aliquoted into pre-chilled microfuge tubes in 50µl amounts and 1µl of each ligation mixture was added to a separate aliquot of cells. The tubes were incubated on ice for 30 minutes, heat shocked at 42C for 45 seconds, then incubated on ice for a further 2 minutes. SOC medium was made up according to Sambrook *et al.* (1989). Briefly, 20g bactotryptone, 5g yeast extract and 0.5g NaCl were dissolved in 950ml water, 10ml of 250mM KCl was added and the pH adjusted to 7.0 with 5N NaOH. A 5ml volume of 2M MgCl₂ was then added and the resulting medium was sterilized by autoclaving. The medium was cooled to 60C, then 20ml of filter sterilized 1M glucose solution was added. SOC medium kept at room temperature (0.45ml) was added to each tube and these were shaken at 225rpm and 37C for 1 hour. These were spread plated onto LB ampicillin agar and incubated for 18 hours, then examined for colony growth. The test and positive control plates had many colonies and the negative control had none when the ligation was deemed successful.

Screening of transformed cells for expression of recombinant HAP: Bacterial colonies from the transformation step were picked using one fresh toothpick per colony, and each used to seed a separate 4ml LB ampicillin broth. The cultures were each given numbers and incubated statically for 3 hours at 30C, then each was split into two cultures of 2ml each; one set was kept at 30C and the other set was immediately incubated at 42C for a further 2 hours. One millilitre volumes of each culture were microcentrifuged at 10000 g for 5 minutes and the pellets suspended in 50µl of SDS-PAGE gel loading buffer. These were boiled and analyzed by SDS-PAGE as described above. For speed, the SDS-PAGE gels were stained with rocking for 30 minutes in an aqueous solution of 40% (v/v) methanol, 10% (v/v) ethanol and 0.1% (w/v) coomassie blue. Gels were then destained in several changes of an aqueous solution of 40% (v/v) methanol and 10% (v/v) ethanol until protein bands could easily be distinguished (about 30 minutes). The expected approximate size of the recombinant protein was previously calculated from the sequence data, and the stained gel was examined for a polypeptide of this size present in the cultures grown at 42C, but absent in cultures kept at 30C. Plasmid DNA was isolated from the 1ml volumes

of each culture remaining using a Recovery plasmid mini prep kit (Hybaid), which was based upon microfuge spin columns. A restriction digest was set up and incubated as above for each plasmid, using 3µl of plasmid DNA, 1µl of OPA+ and 1 unit each of first PstI and then HpaI. The digested plasmids were analyzed by agarose gel electrophoresis as described above and the actual size of plasmids were compared with the predicted size.

PCR and cloning of HAP in the PCR cloning vector pGEM-T and expression vector pGEX-3X

PCR of HAP DNA: Amplification of the whole HAP open reading frame by PCR was repeated, then the product was cleaned and digested with 1 unit of Ssp I using the methods described above, which generated two pieces of HAP DNA: a front half, and a back half (named Fragment 4). Primers were used to amplify this 5' piece of the digested HAP open reading frame (front half) as two overlapping fragments: Fragment "a" (forward: 5'- as forward primer described above [base positions 1-20 on the published sequence], and reverse: 5'- AAGGCCTTTACCCCTAGAGAG-3', [base positions 483-497 on the published sequence]), and Fragment "b" (forward: 5'- AAGGCCTGGCTTGACCTATACAGCA-3', [base positions 288-306 on the published sequence] and reverse:

5'- AAGGCCTAATAGCTGAGGTACCAGAA-3', [base positions 808-825 on the published sequence]). The front half was also amplified as three non-overlapping fragments: front (Fragment 1:- forward: as forward primer described above [base positions 1-20 on the published sequence] and reverse: 5'- AAGGCCTATCCTCTGACACATCAACC-3', [base positions 270-288 on the published sequence]), middle (Fragment 2:-

forward: 5'-GTGGATCCATGGCTTGACCTATACAGCAA-3'[base positions 287-307 on the published sequence] and reverse: 5'-GAGGCCTCTTTACCCCTAGAGAGTCT-3', [base positions 480-498 on the published sequence]) and back (Fragment 3:- forward: 5'-GGGATCCAGGCTGATGGGAATAAGGT-3', [base positions 496-514 on the published sequence] and reverse:

5'-CAGGCCTATTTGCAAAGTCTAGCTTAC-3', [base positions 806-825 on the published sequence]). These primer sets were used to amplify the HAP sequence with flanking BamH1 and StuI restriction enzyme sites so that these inserts would only be able to clone into pGEX-3X in the correct orientation. The constituents and volumes used were the same as described above. Cycling conditions were as described above, except for the annealing temperatures, which were 51, 60, 63, 64 and 64C for Fragments a, b, 1, 2 and 3 respectively. PCR products were analyzed as described above.

Ligation of HAP PCR products with pGEM-T and transformation of JM109

competent cells: The vector for cloning PCR products, pGEM-T, was supplied by Promega. The fragments of HAP DNA amplified above, including the smaller back half fragment (Fragment 4) was ligated into pGEM-T, followed by transformation into *E. coli* strain JM109, using methods described above; the pGEM-T vector required no preparation however, and the JM109 cells used were supplied as competent. Colonies were picked as described above and used to seed separate 4ml LB ampicillin broths, which were shaken at 250 rpm and 37C for 6 hours. One millilitre was removed from each culture, plasmid extracted and purified using a Recovery plasmid mini prep kit (Hybaid); the remaining culture was used to make 20% (v/v) glycerol stocks for long term storage. PCR reactions for HAP gene fragments appropriate to each recombinant clone were used to confirm that the correct inserts were present. Those confirmed to have the correct insert by PCR were sequenced, using the plasmid preparation as template DNA.

Sequencing of pGEM-T clones: Clones were sequenced to check that they were HAP fragments and whether there were any differences in the *S. equi* sequence when compared with the published HAP sequence, which is for *S. equisimilis*. A pre-prepared master mix known as ABI-Prism™ dye terminator cycle sequencing kit, containing Amplitaq DNA polymerase FS was used. Primers SP6 and T7 (Promega), which were complementary to sequences flanking the pGEM-T cloning site were used in two separate sequencing reactions for each clone to obtain sequence from both directions. Four microlitres of master mix was mixed with 1µl of primer at 1.6 pmol/µl, 2µl of water and 3µl of prepared

template DNA. The standard cycle sequencing program was performed on thermal cycler (Hybaid). A 3 minute 95C hot start was followed by 25 cycles of 96C for 30 seconds, 50C for 15 seconds and 60C for 4 minutes, finishing on hold at ambient temperature. Each extension product was added to a separate 1.5ml microcentrifuge tube containing 1µl of 3M sodium acetate, pH4.6 and 25µl of 95% (v/v) ethanol. The tubes were vortexed and incubated at -80C for 10 minutes, then were microfuged at 10000 g for 30 minutes. The ethanol was aspirated from each tube and the pellets rinsed by adding 250µl of 70% ethanol and carefully aspirating. The pellets were dried by incubation at 37C for 30 minutes, then dissolved in 5µl of EDTA formamide buffer, made by dissolving blue dextran in 25mM EDTA, pH8.0 to a final concentration of 50mg/ml, then adding four volumes of formamide. The samples were heated to 90C for 2 minutes to denature the DNA, then 4µl was loaded onto an 4.5% polyacrylamide sequencing gel. The gel was run on an ABI-Prism™ 377 automated sequencing machine according to the manufacturer's instructions (Perkin Elmer). The automated sequencing machine generated an electrophoretogram with each sequence, and this was checked manually to ensure that the computer program did not miss any bases and to choose a base manually, where possible, when the computer algorithm could not select a base. A sequence data analysis package (DNASIS) was used to compare sequence data with the published sequence for HAP (Lansing *et al.* 1993).

Preparation of HAP fragments from pGEM-T for ligation into pGEX: Four mililitre LB ampicillin cultures of each clone (one clone per fragment) shaken at 250 rpm for 6 hours at 37C, were used to seed separate 100ml LB ampicillin broths. These were then shaken at 250 rpm and 37C for 18 hours. Plasmid was extracted and purified from these cultures using a Midi plasmid purification kit (Qiagen), which is based upon the alkaline lysis method described above and is useful for larger plasmid preparations. The plasmids were digested (20µl of each clone) in a 40µl total volume (made up with water), with 2 units of Stu I in the presence of 4µl of 10X OPA+ to a final concentration of 1X, at 37C for 6 hours. Then the Stu I was heat inactivated at 37C for 15 minutes and the sizes of digested plasmids were checked with those predicted by analysis of 10ul of each plasmid

on an agarose gel as described above for PCR products. The digestion of remaining plasmid was completed by adding 2 units of BamHI and 1.2 μ l of 10X OPA+, since BamHI requires a final concentration of 2X OPA+ for optimal activity. The mixture was incubated for 18 hours, then the enzyme inactivated as above. The plasmids were digested with these enzymes to generate a blunt and a sticky end, such that inserts could not be cloned in the wrong orientation.

The HAP fragments were recovered from the digest mixture by electrophoresis in low melting point agar. One 1.5% (w/v) low melting point agarose gel for each insert was loaded in all wells except the outer two wells, with digested DNA mixed with an equal volume of gel loading buffer. The outer two wells contained 2 μ l of 1Kb molecular weight standard and a 2 μ l amount of the relevant digested DNA. The gel was run at 65Volts for 75 minutes, then after electrophoresis, the standard and 2 μ l amounts of clones were cut away and stained in ethidium bromide. The stained part of the gel was reassembled with the unstained part on a UV transilluminator. The region of unstained gel corresponding to the stained HAP fragment was then cut out and purified from the agarose using a Qiaex II DNA extraction kit for agarose gels (Qiagen). The concentration of the purified fragments were determined using methods described above.

Digestion of pGEX-3X expression vector: Expression vector pGEX-3X, supplied by Pharmacia, enables affinity purification of fusion products by a glutathione S transferase (GST) tag. The vector (2 μ l) was digested in a 20 μ l total volume (made up with water), with 2 units of Sma I in the presence of 4 μ l of 10X OPA+ to a final concentration of 2X, at 37C for 6 hours. Then the Sma I was heat inactivated at 37C for 15 minutes and the mixture allowed to cool before adding 2 units of BamHI. The mixture was incubated for 18 hours, then the enzyme inactivated as above.

Ligation of HAP with pGEX-3X and transformation of DH10B competent cells:

Purified insert from pGEM-T was ligated into restriction digested pGEX vector, followed by transformation into *E. coli* strain DH10B, using methods described above for pGEM-T, except that IPTG and XGAL were present in the LB ampicillin plates at concentrations described above, for selection of colonies containing inserts by blue / white screening. The pGEX-3X vector possesses a beta galactosidase gene, which expresses beta galactosidase in the presence of an inducer such as IPTG. If a galactose analogue such as X-GAL is also present, beta galactosidase converts it into a blue product, resulting in blue bacterial colonies. The pGEX-3X multiple cloning site is within the beta galactosidase gene, so if an insert is present, on induction with IPTG, the expression of beta galactosidase is disrupted and the bacterial colony will remain white. White colonies are selected for screening.

Screening of transformed cells for expression of recombinant HAP or HAP fragments:

Screening for expression of the correct fusion protein was carried out using similar methods to those described for pPL-Lambda, except that expression was induced by the addition of 100mM IPTG to the growth medium, rather than a shift in temperature. Briefly, 2ml of LB ampicillin broth was inoculated with one colony, and shaken at 250 rpm and 37C for 4 hours. Stock IPTG (100mM) was added to a final concentration of 100μM, and grown as before for another 90 minutes. The cultures were placed onto ice for 5 minutes then a 20μl aliquot of each clone centrifuged for 5 minutes at 10000 g. The pellet was resuspended in 10μl of sample buffer and analyzed by SDS-PAGE, as described previously. The presence of an expressed GST recombinant polypeptide of the predicted molecular mass was also confirmed by immunoblotting, using a goat anti GST antibody (Pharmacia) diluted 1:2000 and a horseradish peroxidase labeled anti goat antibody (Sigma) diluted 1:2000. Those fitting these criteria were sequenced as described above, using primers supplied for sequencing from pGEX vectors (Pharmacia).

Purification of HAP fragments expressed as a GST fusion protein of pGEX-3X: One colony was used to seed a 10ml culture, which was grown statically for 18 hours at 37C. This was used to seed 100ml of medium, which was shaken at 200 rpm and 37C for 4 hours, after which 100mM IPTG was added and the incubation continued for a further 90 minutes. The bacteria were pelleted by centrifugation at 8000g and 4C for 10 minutes, then resuspended in 50ml of ice cold PBS. The cells were kept on ice and sonicated in short bursts of 30 seconds using a micro tip until the cell suspension had cleared. An aqueous stock solution of 20% (v/v) triton-X100 was added to the sonicate at a final concentration of 1% (v/v) and the mixture incubated at room temperature with gentle mixing for 30 minutes. This was centrifuged at 10000g and 4C for 10 minutes, then the supernatant kept on ice until it could be loaded onto a 200 μ l glutathione sepharose 4B column. The column was prepared by washing ten times with 200 μ l volumes of PBS. The supernatant fraction was applied to the column, then the column was washed with two 2ml volumes of PBS. The GST fusion protein was eluted by applying 200 μ l of 5mM glutathione in 50mM Tris-HCl, (pH8.0). Aliquots of the column fractions were analyzed by SDS-PAGE as described above.

S. zooepidemicus intraperitoneal challenge of mice immunized with recombinant HAP

Recombinant HAP was tested for the ability to reduce mortality and morbidity in a murine intraperitoneal challenge model for *S. zooepidemicus*. Twenty mice were bled as below and distributed randomly amongst the groups. Each recombinant HAP GST fusion protein (Figure 14) was mixed with an equal volume of Freund's Incomplete adjuvant (FIA), to make 5 μ g per dose, and 0.1 ml amounts were administered intraperitoneally to groups of 20 female CBA/CA mice. These were: i) Fragment 1; ii) Fragment 2; iii) Fragment 3; iv) Fragment 4; v) Fragments 1, 2 and 3 and vi) Fragments 1,2,3 and 4. Twenty mice were not vaccinated with anything, 10 mice were given GST in FIA and 10 mice were given FIA only; these mice were combined as a control group in the analysis of results. Mice were boosted 28 days later, and bled at 39 days, then at 40 days, the mice were each challenged intraperitoneally with 5×10^7 cfu of *S. zooepidemicus*. Mice were examined every 4 hours for signs of malaise, slow movement and staring coat, to gauge when euthanasia should be

employed to provide a humane endpoint; the time elapsed between challenge and death was noted. The surviving mice at 50 hours post challenge (controls and the groups with a significantly different survival curve) were examined post mortem and the livers were aseptically removed, ground in a pestle and mortar with PBS and sterile sand, and serial dilutions of these were spread onto blood agar plates which were incubated for 18 hours. The number of colonies were counted and viable counts were calculated. Mice used in the *S. zooepidemicus* challenge were bled before both immunization and challenge, as described in Chapter 4.

Rabbit antisera

Six month old, specific pathogen free, female, New Zealand White rabbits (Charles River), were used to raise antisera to HAP in purified and recombinant forms (see below). Purified HAP vaccine was prepared by boiling 0.5 ml of the pooled HIC column fractions 2 and 3 in 0.5 ml of sample buffer containing marker dye for 5 minutes. This was followed by SDS-PAGE separation, with a 7.5% (v/v) polyacrylamide separating gel and a 4% (v/v) stacking gel set without a comb, in a Protean II apparatus (BioRad) and a constant current of 25mA at room temperature until the dye front reached the bottom of the separating gel. The gel was silver stained as above, then 53 and 55KDa bands were cut out individually, broken up in 2 ml of sterile distilled water and blended with an equal volume of FIA. 20µg of recombinant HAP was blended with an equal volume of FIA. Five 0.2ml volumes of each preparation were given sub-cutaneously to separate rabbits and after 4 weeks, without adjuvant. An additional booster inoculation of 1ml total in FIA administered as above was given at 77 days for the purified preparations. Pre-immunization and 2 weeks post immunization sera were separated from blood taken from the peripheral ear vein.

Opsonisation and capsule formation inhibition testing

The *S. equi* strain was streaked in a straight line across the centre of a blood agar plate. Sterile blotting paper cut into 0.5cm x 2cm strips were soaked with either 250µl of PBS as a negative control, hyaluronidase (Sigma) dissolved in PBS to 3mg/ml (w/v) as a positive

control, pre-immune, or immune rabbit serum described above (heated to 56C for 30 minutes). The strips were then placed at right angles across the bacterial inoculum and the plate was incubated for 18 hours, at 37C. The resulting mucoid streak of growth was examined for any changes in encapsulation around the blotting paper strips. The antisera were also tested for ability to opsonise *S. equi*, using methods described by Chanter *et al.* (1994).

Characterization of purified and recombinant HAP and antibody responses by immunoblotting and dot blotting

Gels for immunoblotting were prepared by boiling 25µg of recombinant HAP, or pooled HIC column fractions 2 and 3 (purified HAP), in an equal volume of sample buffer containing marker dye and SDS, followed by SDS-PAGE, with a 7.5% (w/v) polyacrylamide separating gel and a 4% (w/v) stacking gel in a Protean II apparatus (BioRad) and a constant current of 25mA at room temperature until the dye front reached the bottom of the separating gel. Polypeptides separated by SDS-PAGE were electrophoretically transferred to nitrocellulose and processed as described previously, in Chapter 3.

The reactivity of rabbit antisera and murine challenge plasma with native and recombinant HAP was analyzed by dot blotting. Native HAP applied as 10µl of pooled HIC column fractions 2 and 3 per dot and 10µl of 10µg/ml recombinant HAP applied to a final concentration of 100ng per dot were transferred onto nitrocellulose (Schleicher and Schuell) using a BioDot apparatus (BioRad) according to the manufacturer's instructions. Blots were detected as described above, with pre-immune and immune rabbit, or mouse sera, followed by their corresponding HRP-conjugated immunoglobulin (Sigma). Each antiserum/ plasma was diluted in PBS-T alone, plus an aliquot was absorbed with 5µg/ml GST, and another with 10µg/ml native or recombinant HAP fragments, at 37C for 1 hour, with mixing.

Statistics

Differences in proportions of immunized and control mice affected by challenge were tested by Fischer's Exact test, survival curves were compared by Logrank test and numbers of bacteria in the livers of surviving mice were compared by the Mann-Whitney test (to account for a non-Gaussian distribution created by zero values) using the programs of Graphpad Prism 2.0 (Cherwell Scientific).

Results and discussion

Detection of the hyaluronate associated protein (HAP) and gene in *S. equi* and

S. zooepidemicus

Hyaluronate and associated proteins, precipitated from *S. equi* culture supernatant by cetylpyridinium chloride and fractionated as the unbound material from hydrophobic interaction chromatography (hydrophilic fraction), contained polypeptides of 53 and 55KDa detected by silver staining of an SDS-PAGE gel (Figure 15).

Polymerase chain reaction using primers derived from the published sequence for the HAP from *S. equisimilis* (Lansing *et al.* 1993), with 5' Stu I digestion sites for cloning in frame into the Hpa I site of the expression vector pPL- Lambda, yielded a product of approximately 1.7 Kb from *S. equi* and *S. zooepidemicus* DNA. Digestion of the fragment with Pst I yielded two fragments of the sizes predicted for the amplified HAP gene. Too little protein was present to measure the concentration by available methods but rabbits immunized with the purified material produced antibodies reactive with recombinant HAP detected by dot blotting (Figure 16).

Cloning and expression of the HAP gene from *S. equi*

The PCR product from *S. equi* ligated into pP_L-Lambda and transformed into *E. coli* N4830-1, was stable when cloned in both orientations, as revealed by product sizes produced by double digestion with Pst I and Hpa I, so long as the incubation temperature for the culture was below 31C. Subcultures of recombinants with the insert in the correct orientation at 42C, a temperature which inactivates the cI857 repressor of the P_L promoter, either did not grow or grew but did not produce any additional protein of the size expected of a HAP/N- protein fusion and contained a plasmid of reduced size. There was no effect at 42C on the growth or plasmid size of recombinants with the insert in the wrong orientation. These results suggested that HAP was so toxic in *E. coli* that transcription and translation failed before the culture died, or deleted the plasmid, although this is a tightly controlled expression system. Failure to clone the HAP gene in pP_L- Lambda stimulated

the cloning of sub-domains into the expression plasmid pGEX-3X in an attempt to produce immunogenic fusion proteins lacking toxicity for *E. coli*. The amplified HAP gene was digested with Ssp I and Stu I (site upstream of the gene generated by the forward primer used above) yielding two major fragments (“a” and “b”) corresponding with residues 1 to 275 and 276 to 480 which were cloned into the Sma I site of pGEX 3X to give in frame fusions. Both of the large fragments were cloned but digestion of the plasmids from several induced recombinants revealed that the full inserts were not present, suggesting that there was still retention of biological activity and hence toxicity for *E. coli*. The front half was cloned in three separate pieces, the expression of the front polypeptide (Fragment 1) and back polypeptide (Fragment 3) was achieved and the sequences were similar to *S. equisimilis*, but the middle polypeptide (Fragment 2) produced only one clone containing insert, for which there was a frame shift approximately halfway through the coding region. There were a few base substitutions at the third position in the codon for *S. equi* HAP, as compared with that of *S. equisimilis*, which did not alter the amino acid sequence, and some single base differences base. See Figures 21-23 for the sequence of *S. equi* HAP deduced from sequencing HAP fragment pGEX clones.

Several recombinants with the latter half of the HAP gene had inducible expression of a GST fusion protein of the size expected (approximately 50 KDa) for the cloned fragment as a fusion product. One of the recombinants was selected for production of Fragment 4 protein and the purified protein was used to produce rabbit antibodies that reacted in dot blots of the recombinant and purified HAP (Figure 17).

In vitro activity of antibody to recombinant HAP

Antiserum to recombinant HAP (Fragment 4) did not possess any detectable opsonic activity for *S. equi*. However, when the antiserum was soaked into filter paper strips placed across a streak of *S. equi* inoculum on a blood agar plate and incubated for 18 hours, where growth approached the strip, there was a distinct loss of glossy colonial appearance, which was consistent with the diminution of capsulation. A similar effect was

noted when hyaluronidase, but not when pre-immune serum, was used to substitute the antiserum.

Protection of mice against *S. zooepidemicus* challenge by immunization with recombinant HAP

Up until 27 hours after intraperitoneal challenge with *S. zooepidemicus*, CBA/CA mice immunized with the back half of recombinant HAP had a significantly greater survival rate than control mice ($p = 0.012$, Logrank test) and the proportion of survivors was significantly larger ($p = 0.02$, Fischer's exact test), whilst mice immunized with the other fragments did not (Figure 18). However, survival for the HAP Fragment 4 group was only significant up to 27 hours and after that, significance was lost. The survival rates for all groups were not significantly different from the control group at 50 hours after challenge by Fisher's Exact Test; it was 35% for control groups and between 35 and 55% for all test groups. For survival data, see Figures 24 and 25.

Although the back half of HAP was not toxic to *E. coli*, which could indicate a lack of biological activity, it is possible that antibody bound to the capsule of *S. zooepidemicus*, opsonising and hence encouraging clearance by host phagocytic cells. It is interesting that fragments 1, 2 and 3 were not protective in the mouse given that they probably contained the biological activity, if this is indicated by toxicity for *E. coli*. It is possible that they could be protective for a different animal model, or that the toxicity resided in the part of Fragment 2 which could only be cloned with a frame shift in the base sequence.

Fifty hours after challenge, 7 of 20 controls and 11 of 20 immunized mice survived, but the coats of the controls appeared a little more ruffled and they were slower moving than the immunized mice. The number of bacteria in the livers of the survivors, identified as *S. zooepidemicus*, was determined at post mortem examination (Figure 19). In the Fragment 4 immunized mice there was a mean of 1.51×10^2 cfu *S. zooepidemicus* /g of liver whereas the controls had a mean of 6.3×10^4 cfu /g ($p = 0.0003$, Mann-Whitney test). The numbers of streptococci in the livers of mice immunized with Fragment 4 were concentrated on to

the exclusion of other groups because there was a lack of significant difference in the survival curves of the other groups. The noticeably different clinical appearance between the Fragment 4 immunized and control groups prompted the investigation of number of streptococci in the livers of survivors, but upon reflection, it would be advisable to repeat the experiment and look at the livers of the mice from the other groups too.

Pre-immunization mouse sera did not react with any of the recombinant HAP protein fragments, but pre-challenge sera (i.e. post immunization) from mice given fragment 4, or GST showed a reaction which could be removed by absorption with fragment 4 (Figure 20). Most of the other immunized mouse sera had some antibody to the immunizing fragment, although the absorptions were less convincing, suggesting the presence of non-specific antibody. These blots indicated that the antibody response to fragments 1, 2 and 3 was weaker than the response to the back half, although a combination of all four fragments did not protect perhaps because of antigenic competition. This idea could be tested using an ELISA developed for these fragments.

Figure 15. Fragments of HAP amplified by PCR from *S. equi* DNA. Numbers refer to the length of the amplified *S. equi* sequence (in Base Pairs).

* Refers to restriction enzyme digestion.

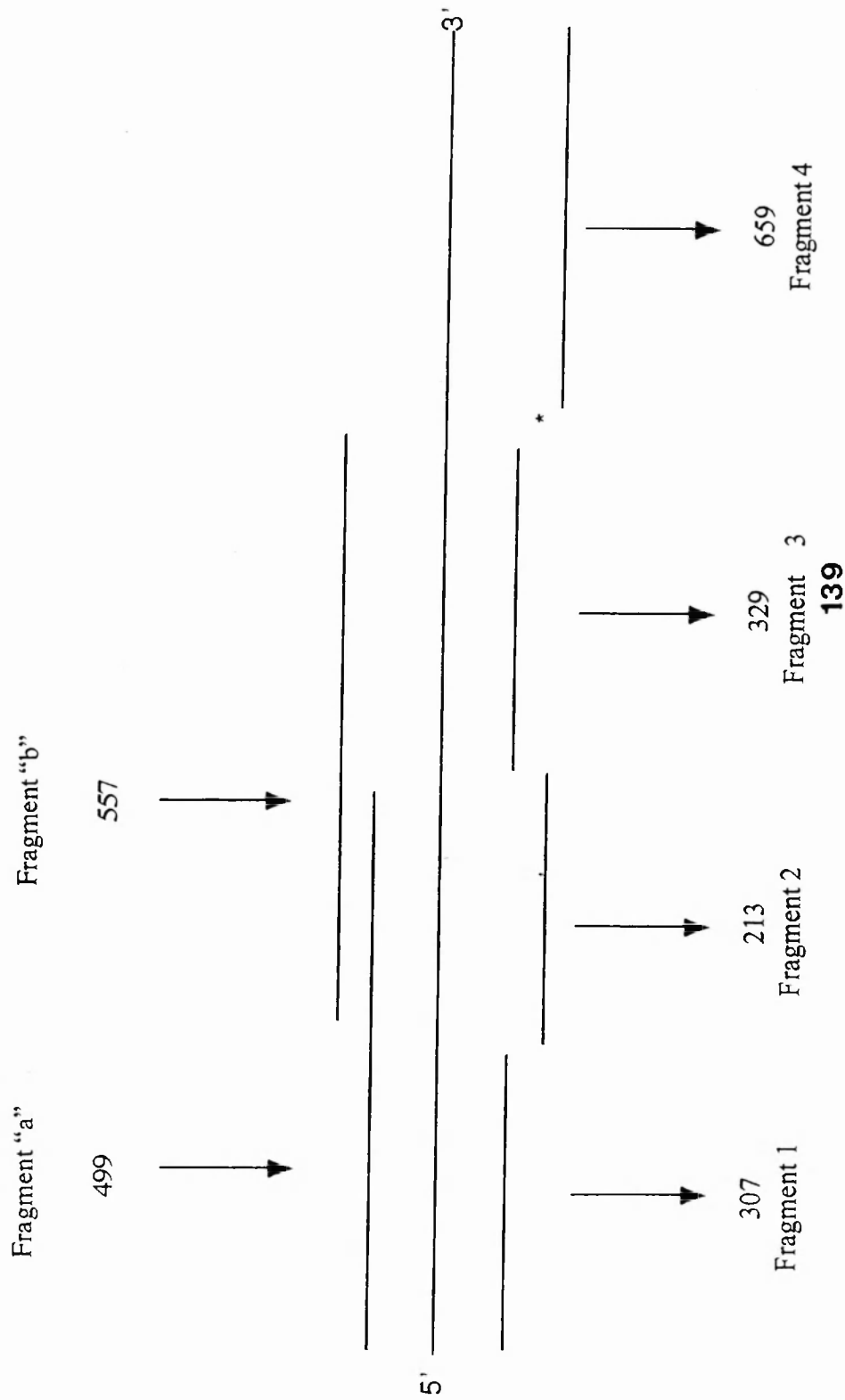


Figure 16. Silver stained SDS-PAGE of HAP precipitated from *S. equi* culture supernatant by cetylpyridinium chloride and fractionated as the unbound material from hydrophobic interaction chromatography.

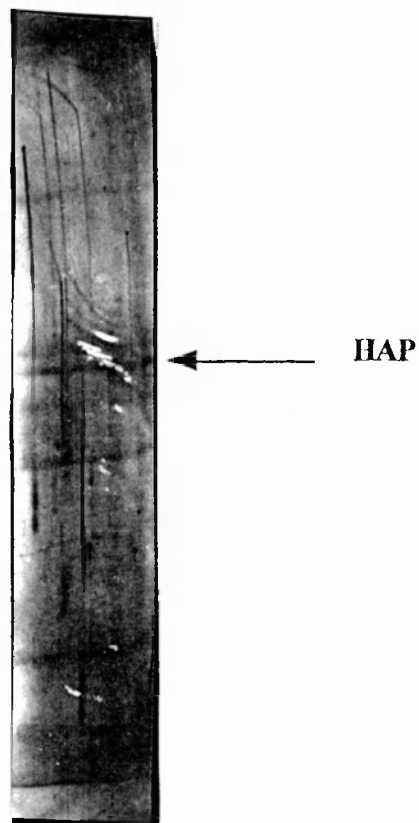


Figure 17. Reactivity of rabbit antisera to HAP precipitated from *S. equi* culture supernatant by cetylpyridinium chloride and fractionated as the unbound material from hydrophobic interaction chromatography, with dot blots of recombinant HAP.

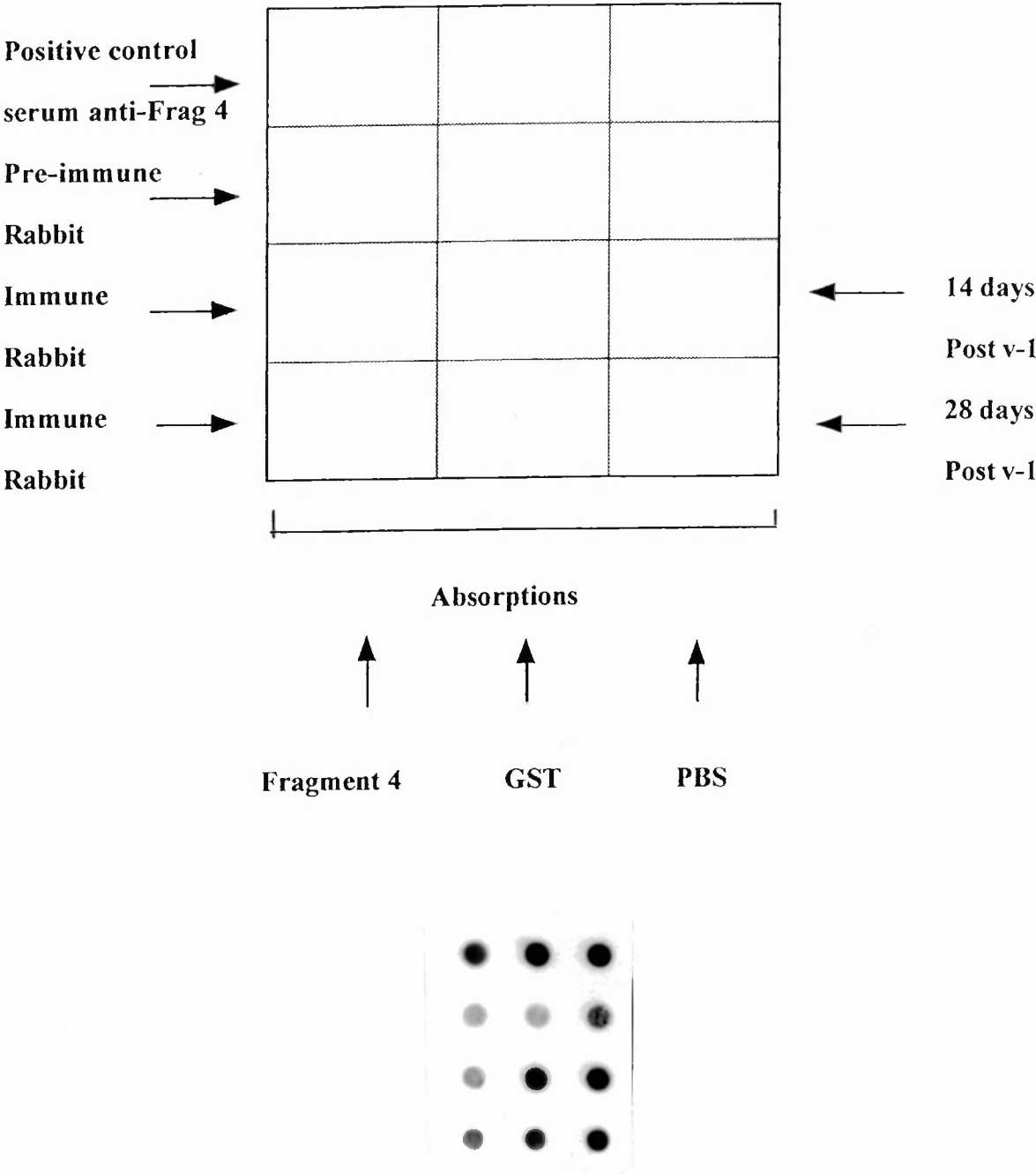
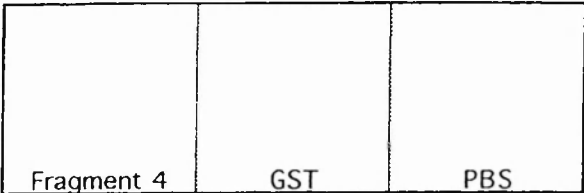


Figure 18. Reactivity of rabbit antisera to recombinant HAP with dot blots of HAP precipitated from *S. equi* culture supernatant by cetylpyridinium chloride and fractionated as the unbound material from hydrophobic interaction chromatography.



Absorptions

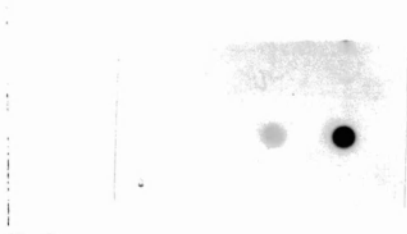
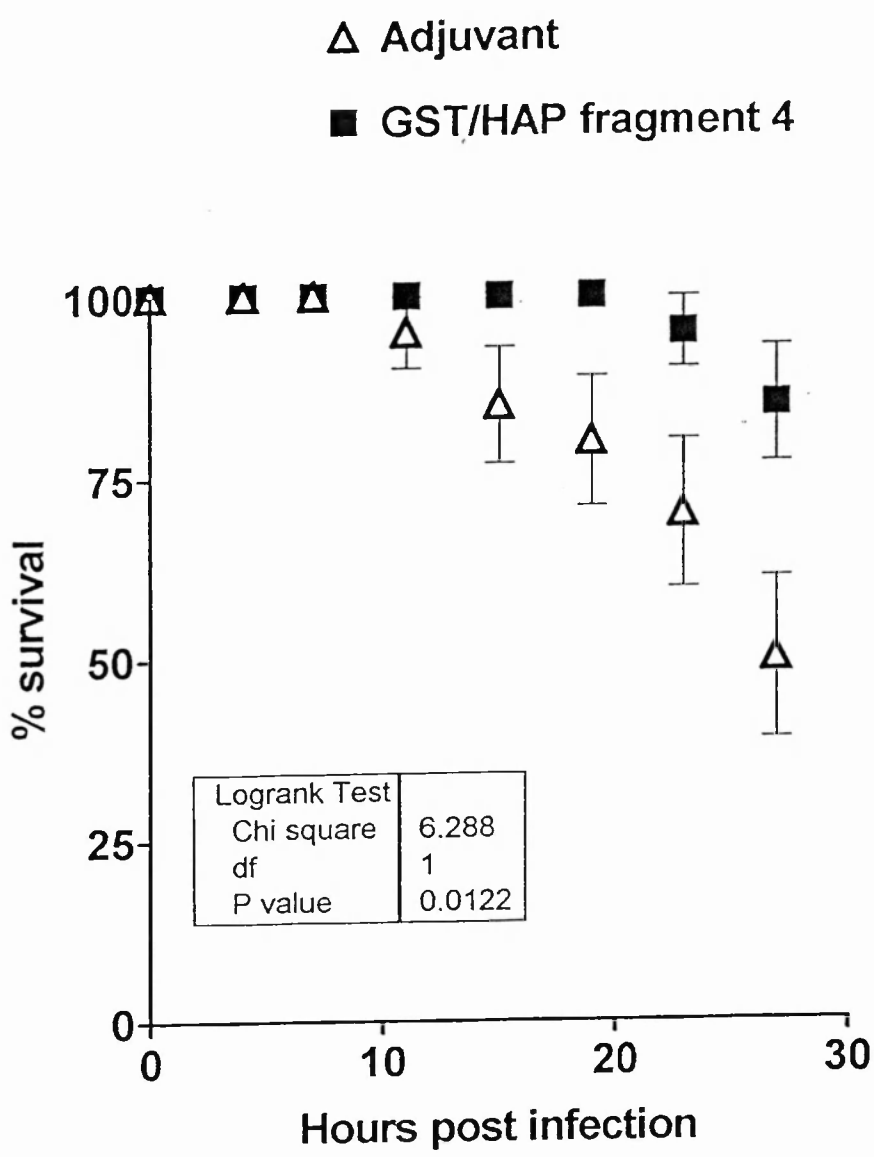


Figure 19

Survival of mice immunised with recombinant hyaluronate associated protein (fragment 4 GST fusion) given *Streptococcus zooepidemicus* by parenteral challenge

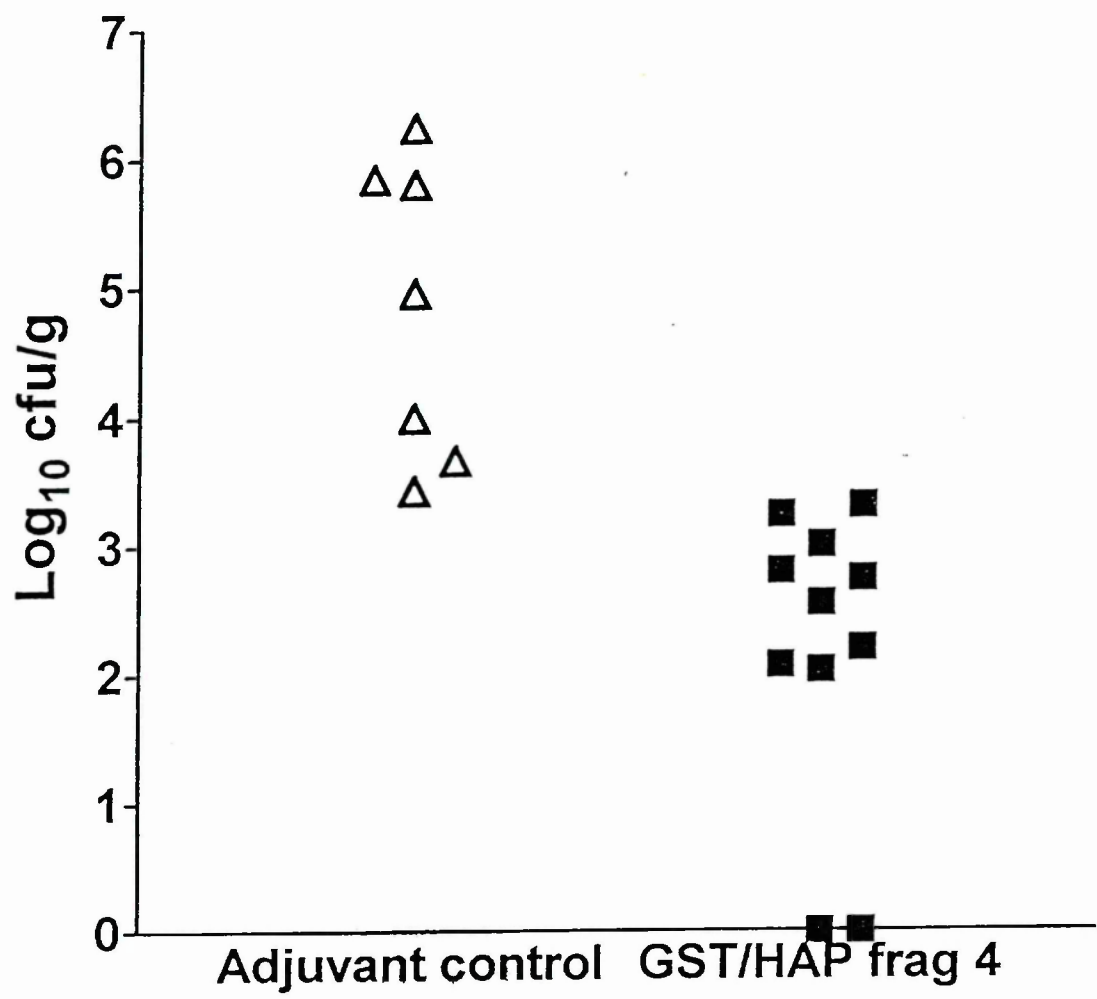


Survival upto 27 hours post infection

Fisher's exact test	
P value	0.0407

Figure 20

Number of *S.zooepidemicus* in the livers
of survivors 50 hours after parenteral challenge of mice
immunised with recombinant hyaluronate associated
protein (fragment 4 GST fusion)



Mann Whitney test	
P value	0.0006
Exact or approximate P value?	Gaussian Approximation
P value summary	***
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	105 , 66
Mann-Whitney U	0.0000

Figure21. Reactivity of mouse sera with dot blots of recombinant HAP.

Absorptions			Immunisation
Fragment 4	GST	PBS	
			Fragment 4
			Adjuvant only
			GST

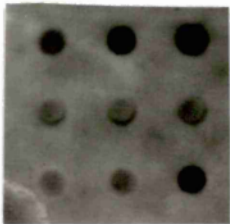


Figure 23. Alignment of *S. equi* HAP Fragment 2 sequenced from pGEX-3X, with the *S. equisimilis* sequence (Lansing *et al.* 1993).

PGEX ATC CAA CGT CGT GGG

S. equisimilis

AT GGC TTG ACC TAT ACA GCA

S. equi ATC CAA CGT CGT GGG ATC CAT GGC TTG ACC TAT ACA GCA

Primer

AT GGC TTG ACC TAT ACA GCA

ACC CTG CGA CAT GGC TTG AAA TGG TCT GAT GGT AGT GAT CTA

ACC CTG CGA CAT GGC TTG AAA TGG TCT GAT GGT AGT GAT CTA

A

Glu

ACA GCA GAG GAC TTT GAG TAC AGT TGG CAG CGA ATG GTC GAT

ACA GCA GAG GAC TTT GTG TAC AGT TGG CAG CGA ATG GTC GAT

Val

*

- CT AAG ACA GCC TCA GAG TAC GCT TAC TTG GCA ACT GAG TCA

CCT AAG ACA GCC TCA GAG TAC GCT TAC TTG GCA ACT GAG TCA

Val

CAT GTG AAA AAC GCA GAG GAC ATT AAT AGC GGG AAA AAT CCT

CAT CTG AAA AAC GCA GAG GAC ATT AAT AGC GGG AAA AAT CCT

Leu

GAT CTA GAC TCT CTA GGG GTA AAG

GAT CTA GAC TCT CTA GGG GTA AAG

A GAT CTG AGA GAT CCC CAT TTC

- Frame shift

Figure 25. The proportion of mice immunised with HAP Fragments 1, 2, or 3, compared with control mice, surviving challenge with *S. equi* over time.

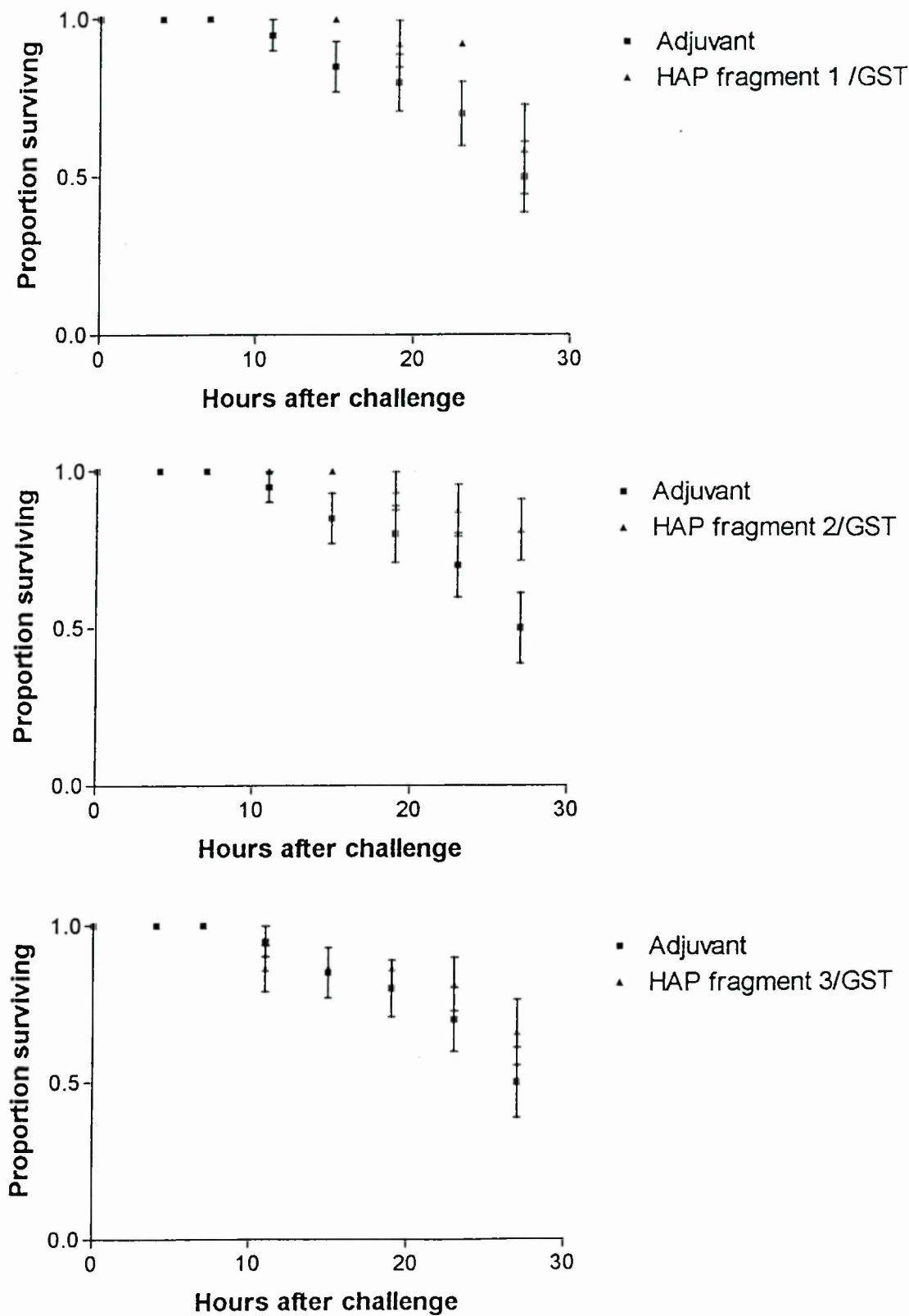
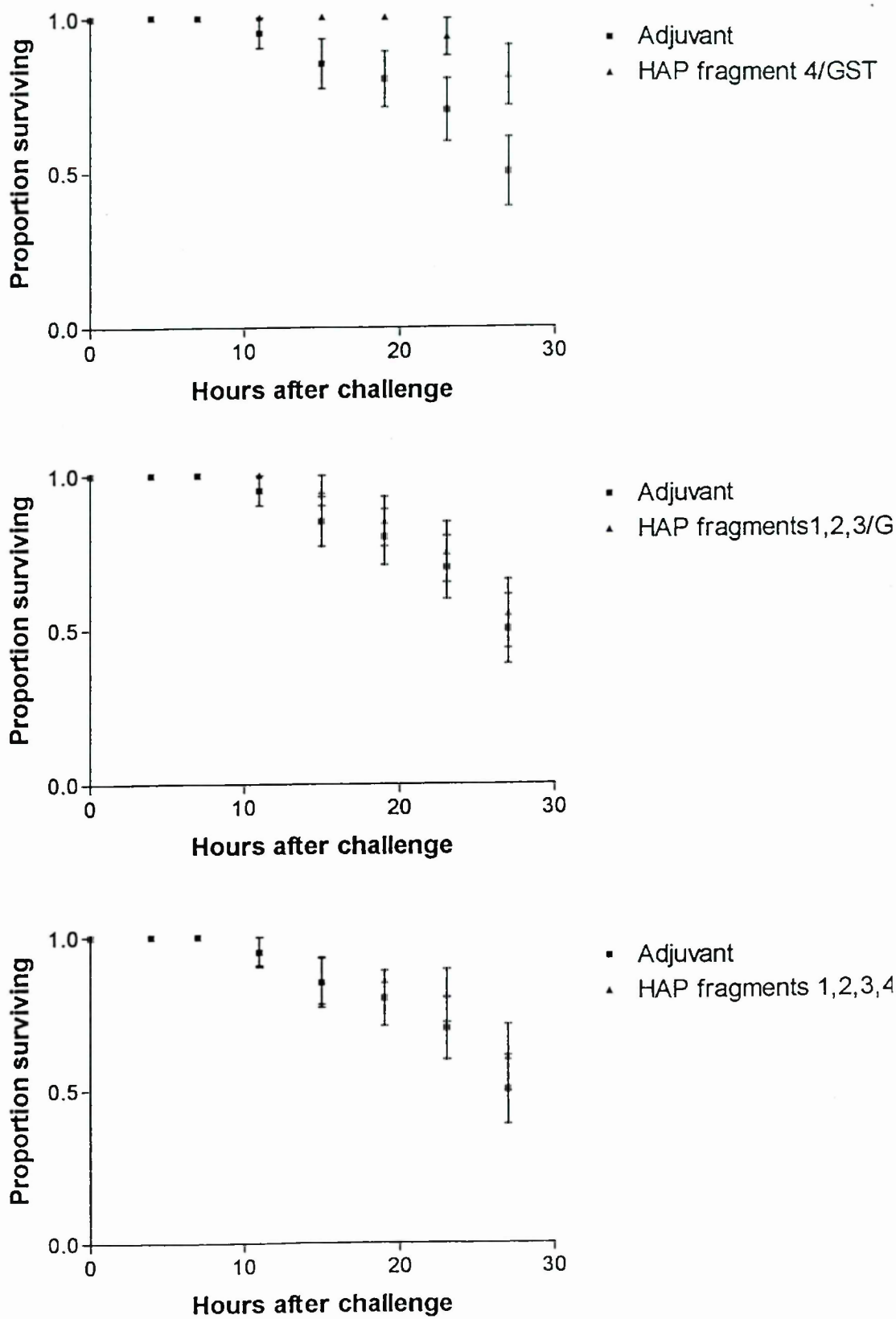


Figure 26. The proportion of mice immunised with combinations of HAP Fragments, compared with control mice, surviving challenge with *S. equi* over time.



6.0 CHAPTER SIX

DEVELOPMENT OF EQUINE RESPIRATORY CHALLENGE MODELS
FOR *STREPTOCOCCUS ZOOEPIDEMICUS* AND
***ACTINOBACILLUS EQUULI*.**

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Objectives

The main objectives of this study were to develop respiratory challenge models in the equine for *S. zooepidemicus* and *A. equuli*, which would fulfil Koch's postulates for these bacteria as pathogens in the equine respiratory tract, using the lowest dose of bacterial culture, and provide models in which to test potential protective immunogens.

Summary.

The development of reproducible equine respiratory challenge models for *S. zooepidemicus* and *A. equuli* in the equine, using non-SPF animals was investigated. Ponies judged to be naive to challenge isolates by immunoblotting at 13 days prior to challenge were split into two groups; one group was challenged with *S. zooepidemicus* and the other group with *A. equuli*. There was a gross pathological and histopathological dose response detected for challenge with both *A. equuli* and *S. zooepidemicus*. Although no live bacteria could be cultured from the lung lesions, immunostaining for bacterial antigen using rabbit antisera revealed its presence. Tracheal washes taken on the day of challenge revealed that the ponies had evidence of bacterial lower airway disease and immunoblotting of bacterial extracts with sera taken on the day of challenge suggested recently acquired immunity, which may have affected their susceptibility to the challenge organisms. If Koch's postulates are to be satisfactorily fulfilled with these bacteria in the lower airway of the equine, specific pathogen free (SPF) animals housed in containment facilities should ideally be used for a reproducible challenge model.

One of the major difficulties presented by a challenge model for these bacteria is that they are frequently encountered by the young animal in sequential episodes of disease. We have hypothesised that the ongoing susceptibility is related to antigenic variation in the surface of M-like proteins of *S. zooepidemicus* and the iron limited proteins of *A. equuli* which are potentially protective determinants. Consequently, the selection of non-immune animals was an essential part of developing a challenge model. In Chapters 3 and 4, tests were developed to assess immune status and these were used in the selection of animals for this study. A terminal study was necessary because bacterial pneumonia in foals is difficult to recognise and assess the severity of clinically, and often does not correlate well with the presence of pulmonary consolidation (Mair, 1989; Koterba, 1984). Another potential difficulty with the equine is that examination of the antibody response to vaccination with *S. pneumoniae* type 3 capsule followed by challenge with a large dose of an 18 hour culture, revealed worse lesions in vaccinated animals than controls and acute respiratory distress indicative of adverse immune reactions. This is contrary to experiences

with mice and man, which show tracheal signs and limited consolidated pneumonia following administration of a large dose of culture. Attempts were therefore made to find the lowest dose of culture which would reproduce disease, giving a low antigenic challenge and therefore the least chance of complicating the model by adverse immune reactions in challenge.

Materials and methods

Animals

Twenty-one ponies from group A, as detailed in chapter 3, were used. Thirteen days prior to challenge with *S. zooepidemicus*, the nasopharynx of each pony was swabbed and tested for the presence of Streptococci and Actinobacillus/Pasteurella species, and influenza. Sera were also taken at the same time and tested for reactivity to extracts of *S. zooepidemicus* and *A. equuli* (see below).

Sera

Ponies were bled 13 days prior to challenge, on the day of challenge and immediately prior to euthanasia. Blood was allowed to clot and sera were collected for immunoblotting, as described in chapter 3.

Western immunoblotting

Immunoblots were made from SDS-PAGE of 100µg of each hot acid extract of *S. zooepidemicus* A-E, or each sodium salicylate extract of *A. equuli* R, S, X and Y, grown with or without 2,2-dipyridyl, as described in chapter 4 and chapter 3, respectively. Pony serum was tested at the pre-challenge, challenge day and post-mortem stages of the experiment. Antibody reactions were recorded and used to decide whether ponies were suitably naive to the chosen isolates prior to challenge, to select a suitable isolate of *S. zooepidemicus* and *A. equuli* for each challenge and to see the effects of challenge on circulating antibody.

Housing and management

Ponies were selected for challenge at the place of supply, an isolated hill farm, where they were kept together in a barn. They were then transported together in a three hour journey one week later and housed three to a stable with straw bedding. They were provided with hay twice per day and water *ad libitum*. Rates of food and water consumption were noted in the settling in period, of one week before the challenge. Any changes in the daily food and water consumption as compared with the settling in period were recorded.

Preparation of inoculum for challenge

Of the extracts of isolates tested by immunoblotting, the pony sera were judged to contain least antibody to *S. zooepidemicus* isolate C and *A. equuli* isolate R, so these were used for the pony challenge. The inoculum was made by growing ten separate 10 ml cultures without 2,2-dipyridyl but with shaking at 37°C. After 4 hours, the cultures were pooled and tenfold serial dilutions to 10^{-2} were made in PBS pre-warmed to 37°C. These were transported to the challenge site in a polystyrene container to maintain them at approximately 37°C and 10ml of each was instilled into the trachea of individual ponies using an endoscope. Viable counts were made on an aliquot of the remaining culture after it had been administered to the ponies.

Parameters measured

At the same time each morning, rectal temperatures and number of breaths per minute were taken and the quality of breathing, presence of purulent or clear ocular or nasal discharges, whether ponies were feeding, drinking, or depressed, whether standing or recumbent, were noted. These were monitored at more frequent time intervals dependent on the severity of symptoms. On the day of challenge, tracheal washes were taken: these were fixed with formalin for pathology, mixed with EDTA for cytology, and left plain for bacteriology and virus detection. An aliquot of each tracheal wash was sent to David and Helena Windsor of Mycoplasma Experience, Reigate, Surrey, for the isolation of *Mycoplasma* spp. For the *S. zooepidemicus* challenge, two ponies from each challenge group were slaughtered three days after challenge and the remaining test ponies slaughtered along with two unchallenged control ponies four days after challenge. One of the control ponies was kept for use as a control in the *A. equuli* challenge. Similarly for the *A. equuli* challenge, two ponies from each challenge group were slaughtered three days after challenge and the remaining test ponies slaughtered along with two unchallenged control ponies four days after challenge.

After euthanasia by an overdose of anaesthetic (10ml Somulose) the carcass was weighed, the pluck was removed, heart removed and swabbed for bacteriology, the pluck was

weighed, then to provide lung density measurements, the lung volume measured by displacement of water. Tissue pool was taken for virus and mycoplasma isolation. Bronchoalveolar lavage (BAL) and tracheal wash (TW) samples were taken for histopathology, bacteriology, and virology, then the trachea was weighed to subtract from the pluck weight, to give the lung weight. The lung surface was examined for evidence of pneumonic lesions and representative regions of affected lung dissected out for histology and bacteriology. Tissue pools from each pony, comprising lung, liver, spleen and sub-mandibular lymph nodes were examined for the presence of *Mycoplasma* spp., as above.

Results

Antibodies in the sera of twenty-one ponies 13 days before challenge, to proteins in hot acid extracts of five *S. zooepidemicus* isolates and sodium salicylate extracts of four *A. equuli* isolates grown in iron limited media.

Pony sera taken prior to purchase, were tested for antibody to polypeptides in extracts of each *S. zooepidemicus* isolate, and to IRPs and non-IRPs in extracts of each *A. equuli* isolate separated by SDS-PAGE and transferred to nitrocellulose. There was less antibody to extracts in the sera of these ponies than seen in the serum of an old pony, or with rabbit antisera raised to extracts of isolates for a previous experiment.

For *S. zooepidemicus*, all ponies had antibody to a 52 KDa polypeptide in the extract of isolate A, and 38% had antibody to a 46 KDa polypeptide. There were also traces of antibody to a series of lower molecular weight polypeptides. 90% of ponies had antibody to a 35 KDa polypeptide in the extract of isolate B, and 76% had antibody to a 46KDa polypeptide but only 33% had antibody to a 52KDa polypeptide. Ninety percent of ponies had antibody to a 35 KDa polypeptide in the extract of isolate C, but only 62% had antibody to the 46KDa polypeptide. The same 62% also had antibody to a number of lower molecular weight polypeptides. All ponies had antibody to a 52 KDa polypeptide in the extract of isolate D, and 62% also had antibody to 48,46 and 35KDa polypeptides. Only one pony had antibody to a 52 KDa polypeptide in the extract of isolate E, although 6 of the ponies had antibody to a 48 KDa polypeptide.

Pony sera had antibody least reactive with the extracts of isolates C and E. Isolate C was used in the challenge as all ponies had relatively less antibody to higher molecular weight polypeptides than to extracts of the three other isolates. Isolate E was previously isolated from the premises on which the challenge was carried out, presenting a possible risk of infection prior to challenge, so E was excluded.

All four isolates grown in medium in which iron was limited, then sodium salicylate extracted, produced seven proteins in iron limited conditions which were absent or much

fainter in iron replete conditions (IRPs). The molecular masses of these were as described in Chapter 3. Pony sera contained antibodies which reacted with the IRPs the in extracts of *A. equuli* isolates X and Y. Most of the reactivity for isolate Y was for the 73 KDa polypeptide whereas for isolate X it was for polypeptides of 60 KDa and below. None of the sera had antibodies to the IRPs of extracts of R or S, so isolate R was chosen as the challenge isolate.

Bacteriological and virological testing of nasopharyngeal swabs from twenty-one ponies prior to *S. zooepidemicus* challenge.

No influenza antigen was detected by ELISA from the nasopharangeal swab transport fluid. *S. zooepidemicus* was isolated from nine of the twenty-one ponies but in small numbers and there was otherwise no significant growth.

STREPTOCOCCUS ZOOEPIDEMICUS CHALLENGE

Bacteria isolated from nasopharyngeal swabs of ponies taken immediately prior to challenge with *S. zooepidemicus*

Twelve ponies were swabbed immediately prior to challenge with *S. zooepidemicus* (Table 10). *S. zooepidemicus* was isolated from all but one pony. *B. bronchiseptica* was isolated from eight of the twelve ponies and coagulase positive staphylococcus were isolated from seven of twelve animals. A *Pasteurella* species was isolated from one of the ponies. Non-haemolytic streptococci, coagulase negative staphylococci, bacillus and fungus species were also isolated.

Endoscopy examination and tracheal wash bacteriology, cytology and virology from ponies immediately prior to challenge with *S. zooepidemicus*

Only four of the nine ponies to be challenged had obvious mucopus on endoscopy and in the tracheal washes. There were no visible signs of erythema or oedema of the tracheal wall. Most of the ponies sampled had an inflammation score of 1/2, and had evidence of a predominance of neutrophils or macrophages, thickened or copious mucus. Gram positive cocci associated with squames and /or mucus were seen in four and gram negative cocci in

two of the tracheal washes. Bacteriology of the tracheal washes (Table 11), revealed *S. zooepidemicus*, *B. bronchiseptica* and coagulase positive staphylococci, each in six ponies. The count was greater than 10^4 cfu/ml for all *B. bronchiseptica* isolated, but bacterial counts were lower in some tracheal washes for the other bacteria. A widely variable count of non-haemolytic Streptococci were also isolated from seven of the eight tracheal washes and a high count of *Pasteurella* spp. was found in one. All ponies tested negative for influenza antigens and no *Mycoplasma* spp. were cultured. Taken together, the evidence suggested a mild to moderate bacterial lower airway infection in the ponies immediately prior to challenge.

Clinical signs recorded in ponies challenged with *S. zooepidemicus*

Rectal temperatures (Table 12) for two of the control ponies were 36.5°C and above on the morning of the challenge, but were otherwise lower than this for all ponies throughout the experiment. The mean temperatures of the ponies given a 10^{-2} dilution of culture increased 24 hours after challenge, but decreased for ponies in other groups including the control ponies. By forty-eight hours, the temperatures the 10^{-2} group had stabilised, but continued to decline for those given neat culture, and control groups. Temperatures had stabilised in all groups by 72 hours.

Mean breathing rates (Table 13), were relatively high on the day of challenge for all groups, including controls, but gradually declined as the experiment continued for the group given a 10^{-1} dilution of culture. Groups given neat and 10^{-2} diluted culture had increased breathing rates 24 hours after challenge, but by 48 hours, rates had decreased to lower levels than those measured prior to challenge. All ponies challenged with neat culture and one of the ponies given 10^{-1} were breathing abdominally 24 hours after challenge. By 72 hours, the pony in the 10^{-1} group breathing abdominally 24 hours after challenge, was making a railing breathing noise and the other two ponies in the 10^{-1} group were also breathing abdominally at this time.

Twenty-four hours after challenge, three of the ponies challenged with neat culture showed signs of depression and coughing was heard. This also included the group given the 10^{-2} dilution at 48 hours and to all challenged groups at 72 hours, by which time the group given 10^{-1} of culture dilution were not eating normally. Seven of the twelve ponies had ocular discharge on the day of challenge, which increased to eleven of the twelve 48 hours after challenge and included control ponies. Nasal discharges also peaked at 48 hours but of the five involved, two were control ponies.

Bacteriology and cytology of tracheal wash (TW) and bronchoalveolar lavage (BAL) samples taken post mortem from ponies challenged with *S. zooepidemicus*, and controls

Large numbers of *B. bronchiseptica* and moderate numbers of *S. zooepidemicus* and staphylococcus spp., were isolated from both TW and BAL samples of ponies (Table 14). This indicated a *B. bronchiseptica* infection. Samples were clear or translucent with variable flocculation. Eight of the inflammation scores were 1/3 and three (two from separate challenge groups and one of the unchallenged ponies) had scores of 0/3. The predominant cell types found were neutrophils and epithelial cells, and mucus was generally eosinophilic, none of which appeared to be related to challenge dose. Gram-positive and negative bacteria were associated with squames and in some cases mucus in uninfected controls as well as challenge ponies.

Pathology and bacteriology of tissue samples taken post mortem from ponies challenged with *S. zooepidemicus*, and controls

Histological signs of multifocal lobular pneumonia were found in all three ponies given 10^{10} cfu of culture (Table 15), two of the three given 10^9 cfu (Table 16) and two of the three given 10^8 cfu of culture (Table 17). This was not found in two of the test ponies or the uninfected controls (Figure 18). Lateral cardiac and accessory lobe regions were consistently affected. The most extensive lesions were found in ponies labelled 14 and A (10^{10} cfu) and 9 (10^9 cfu).

The controls and two test ponies 8 and 13 revealed evidence of pre-existent lower airway inflammatory changes and minor parenchymal lesions. Parasitism may have contributed to an eosinophil response seen in some ponies. No significant changes were seen in the upper respiratory tract, except for a tracheitis in pony 13 (10^9 cfu) and there was no evidence of abscessation of respiratory tract lymph nodes. Bacterial colonies were not commonly evident in lung sections but immunostaining detected the presence of lesion-associated bacterial antigen.

Ocular discharge from control ponies contained predominantly *S. zooepidemicus* and *Staphylococcus* spp. However, very few *S. zooepidemicus* were recovered from the tissues of the test ponies. For the ponies given 10^9 cfu, there were moderate to large numbers of *B. bronchiseptica* in the lymph nodes and normal lung. One lesion contained large numbers of *Nocardia* spp. Moderate to large numbers of *B. bronchiseptica* and smaller numbers of *Staphylococcus* spp were also cultured. Ponies given 10^{10} cfu had *B. bronchiseptica* and *Staphylococcus* spp. present in lesions and one of the ponies had small numbers of *S. zooepidemicus* in the bronchial lymph node. The apparent dose response and immunoperoxidase positive staining bacteria present in sections of tissue suggests that *S. zooepidemicus* was associated with the lung lesions. *Gasterophilus* spp. and *Parascaris equorum* were found in the intestines of all ponies. No *Mycoplasma* spp. were cultured from tissue pools, or influenza antigens detected.

Antibodies of twenty-one ponies immediately prior to and three/four days after challenge, to proteins in hot acid extracts of five *S. zooepidemicus* isolates.

The antibodies in the sera of most of the ponies reacted more strongly and to a wider range of polypeptides in all of the extracts than seen in the sera taken 13 days before challenge. Also the strengths of the reactions were more consistent between ponies. Antibodies in sera taken on the day of euthanasia were not appreciably different to those in sera taken on the day of challenge.

ACTINOBACILLUS EQUULI CHALLENGE

Bacteria isolated from nasopharyngeal swabs of ponies taken immediately prior to challenge with *A. equuli*

Nine ponies were swabbed immediately prior to *A. equuli* challenge (Table 19). *S. zooepidemicus* was isolated from all but one pony, *B. bronchiseptica* was isolated from seven out of nine ponies and coagulase positive *Staphylococcus* spp. were isolated from 2/9 animals. *Pasteurella* species were isolated from 4/9 ponies. Non-haemolytic streptococci, coagulase negative staphylococci, bacillus, and fungus species were also isolated. *S. equisimilis*, a beta-haemolytic streptococcus and coliform were each isolated from one of the ponies.

Endoscopy examination and tracheal wash bacteriology, cytology and virology from ponies immediately prior to challenge with *A. equuli*

Only three of the ten ponies had obvious mucopus on endoscopy and the tracheal washes were mostly clear or translucent with some flocculation. There were no visible signs of erythema or oedema of the tracheal wall. Five of the ponies sampled had an inflammation score of 1/3, two had 2/3 and one had a score of 0/3. Six of the washes had a predominance of neutrophils or macrophages, and most had eosinophilic mucus, of which three were thickened or copious. Gram positive cocci associated with squames and /or mucus were seen in six and gram negative cocci in five of the tracheal washes. *S. zooepidemicus* and *B. bronchiseptica* were isolated from seven tracheal washes and Coagulase positive staphylococci in smaller amounts and variable counts of non-haemolytic streptococci were isolated from five and six ponies respectively (Table 20). *Pasteurella* spp. were isolated from three of the tracheal washes. Counts of greater than 10^4 cfu/ml were found in only half of the samples, which included four with *B. bronchiseptica*, two with *S. zooepidemicus*, and one with *Pasteurella* spp. All ponies tested negative for influenza antigens and no *Mycoplasma* spp. were cultured. It is likely that the pre-challenge respiratory bacterial infection seen in ponies challenged with *S. zooepidemicus* seven days previously, was also affecting the ponies about to be used for

the *A. equuli* challenge. However, the evidence suggested that the infection was less severe at the time of tracheal wash sampling than that seen in ponies the previous week.

Clinical signs recorded in ponies challenged with *A. equuli*

Mean rectal temperatures (Table 22), were relatively stable throughout the experiment. One pony in the group given a 10^{10} of culture had a temperature of 37°C on the morning of the challenge, and one pony in the group given 10^8 had a temperature of 36°C immediately prior to challenge, which rose to 37°C 24 hours later, then subsided.

Mean breathing rates (Table 22), had increased 24 hours after challenge for all groups, including controls, then declined although there was no clear pattern. Two of the three ponies given 10^{10} of culture were breathing abdominally 24 hours after challenge and ponies in the other test groups were breathing slightly abdominally. Forty-eight hours after challenge, the quality of breathing was restored with the exception of one pony in the group given 10^9 of culture, which was still breathing abdominally. By 72 hours, the group given 10^{10} of culture were again breathing abdominally, as was the pony in the group given 10^9 , which continued until post mortem 3-4 days after challenge.

Five hours after challenge, the group given 10^9 diluted culture were sweating. Twenty-four hours after challenge, all of the test ponies had signs of depression and coughing was heard from the group given 10^{10} culture. All test ponies were eating and drinking much less. After 48 hours, all ponies were much brighter and higher levels of eating and drinking were resumed, but they were still quieter than the controls.

Nasal discharges were only present in two of the ponies on the day of challenge. This increased to six 24 hours after challenge, but the majority resolved by the end of the experiment.

Cytology and bacteriology of tracheal wash (TW) and broncho-alveolar lavage (BAL) samples taken post mortem from ponies challenged with *S. zooepidemicus*, and controls

Large numbers of *B. bronchiseptica* were isolated from most of the ponies. *Pasteurella* spp., *S. zooepidemicus* and *Staphylococcus* spp. were isolated from some ponies (Table 23). Samples were clear with variable amounts of flocculation. Inflammation scores were either 1/3 or 0/3 for unchallenged controls and challenged ponies, in no particular pattern. The predominant cell type were epithelial, apart from two of the ponies in groups given 10^{10} and 10^8 cultures, where macrophages predominated, which may be indicative of resolving infection. Mucus was scant and sometimes eosinophilic.

Pathology of the lungs and bacteriology of tissue samples taken post mortem from ponies challenged with *A. equuli*, and controls

The three ponies given 10^{10} cfu of culture demonstrated partial to complete consolidation of the left cardiac regions (sometimes extending to involve the cranial part of the left diaphragmatic region) and right accessory lobes of the lungs. The consolidated areas of lung were characterised histologically by alveolar collapse and interstitial infiltration by a mixed population of macrophages, lymphocytes, neutrophils and eosinophils, with plugging of interposed terminal bronchioles by inspissated exudate. Small foci of alveolar septal necrosis were noted within these pneumonic lesions in pony 16. The lesions in this group of ponies given neat culture were defined as foci of lobular bronchiolar-interstitial pneumonia.

In the group given 10^9 cfu of culture ponies 15 and 2 demonstrated minor congestion or consolidation of the left cardiac regions and right accessory lobes of the lungs. These consolidated areas of lung in pony 15 were characterised histologically by alveolar collapse and interstitial infiltration by macrophages and lymphocytes accompanied by small numbers of neutrophils and eosinophils, and thereby resembled minor lesions of the type seen in ponies given neat culture, whereas the lesions in pony 2 were more typically eosinophil-dominated, and occasional interposed bronchi and bronchioles in this animal were plugged by mucus or eosinophilic exudate. The third pony in this group did not

demonstrate gross pulmonary consolidation, but histological examination of specimens of lung also revealed plugging of bronchioles by eosinophilic exudate and there were tiny foci of alveolar collapse and infiltration by eosinophils, macrophages and neutrophils. A mild subacute tracheitis was recognised microscopically in ponies 5 and 15. Lesional bacteria were not recognised on Gram-staining of lung sections in the groups given 10^{10} cfu or 10^9 cfu but immunostaining revealed bacterial antigen.

There were no signs of gross pulmonary consolidation in the ponies given 10^8 cfu of *A. equuli*. Histological examination of lung specimens revealed occasional microscopic foci of alveolar collapse and mild interstitial infiltration by macrophages, lymphocytes, and neutrophils in the right cardiac and accessory lobes of pony 21. Both ponies had mild subacute tracheitis.

The two control ponies did not demonstrate gross pulmonary consolidation. No foci of alveolar collapse and interstitial infiltration directly comparable to the pneumonic lesions in the test ponies were recognised on histological examination of lung specimens. Both ponies had plugging of the distal trachea by inspissated mucopus, corresponding to a subacute exudative tracheitis.

All ponies demonstrated some other histological changes in the lungs of greater development and resolution which were considered to be not directly related to challenge having occurred earlier. Bronchial and bronchiole-associated lymphoid nodules were generally prominent, consistent with the age of the animals and often showed follicular or micronodal differentiation. There was variable eosinophilic infiltration of bronchial and bronchiolar mucosa, and some airways were plugged by mucus entrapping eosinophils and macrophages. The latter change was considered likely to reflect a pre-existing airway hypersensitivity or endoparasite reaction. No significant changes were recognised in the upper respiratory tract or respiratory tract lymph nodes of the ten ponies.

No *A. equuli* were recovered from the tissues of the test ponies. For the ponies given 10^{10} cfu (Table 24), there were moderate numbers of *B. bronchiseptica* and non-haemolytic streptococci in the lymph nodes and normal lung as well as the dorsal cardiac congestion found in pony B. Moderate to large numbers of *B. bronchiseptica* were cultured from normal lung of ponies given 10^9 cfu of culture (Table 25), along with some *Acinetobacter*, *Staphylococcus* spp. and non-haemolytic streptococci. *Pasteurella* spp. was cultured from the lesion from pony 2.

Ponies given neat culture had *Pasteurella* spp, *S. zooepidemicus*, *B. bronchiseptica*, *Acinetobacter* spp, *Nocardia* spp and *Staphylococcus* spp. present in lesions and normal lung. Overall, there is a possibility that there was some *Pasteurella* spp. associated with the lung lesions, although a *Pasteurella* spp. was isolated from the bronchial lymph node of one control pony. *Gasterophilus* spp and *Parascaris equorum* were found in the intestines of all ponies. No *Mycoplasma* spp. were cultured from tissue pools, or influenza antigens detected.

Antibodies of twenty-one ponies immediately prior to and three/four days after challenge, to sodium salicylate extracts of four *A. equuli* isolates grown in iron limited media

Isolate R was used for the challenge. Although ponies had little circulating antibody to sodium salicylate extract of dipyrindyl grown R in the month prior to challenge, seven of the ponies had antibody to 73, 65 and 48KDa IRPs on the day of challenge. On the day of euthanasia, all ponies had circulating antibody to the 73 and 65KDa IRPs.

On the day of challenge, there was a big increase in reactive antibody to the extract of isolate S; eight of the ponies had circulating antibody to the 73, 65, 60 and 48 KDa IRPs. It is of interest to note that on the day of euthanasia, there was no detectable antibody in any of the sera to the 73 and 65 KDa IRPs, although reactivity to other polypeptides was still strong.

The antibody reactivity to the extract of isolate X on the day of challenge was stronger than previously, and four of the ponies had antibody to both the 73 and 65KDa IRPs; three other ponies had antibody reactive with only the 56KDa IRP. On the day of euthanasia, the antibody response was stronger and more uniform for all of the ponies, including controls.

The antibody reactivity for the extract of isolate Y was not appreciably different from the previous occasion tested on the day of challenge, with reactivity to some IRPs. The response on the day of euthanasia was again similar but stronger. These results suggest that antibody reactive with extract of the challenge isolate was already on the increase in the days prior to challenge. Immunoperoxidase staining of lung lesions using rabbit antisera raised to isolate R suggested that bacteria were present.

Table 10. Identification of bacteria isolated from nasopharyngeal swabs of ponies immediately prior to challenge with *S. zooepidemicus*.

Inoculum	Pony	Bordetella bronchiseptica	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
10-2	12	/	/		/	/	
	20	/	/		/	/	
	8		/	/	/	/	
10-1	9	/	/		/	/	Fungus spp.
	13		/			/	Bacillus spp.
	6		/		/	/	Fungus & Bacillus spp.
Neat	14	/	/		/	/	Fungus & Bacillus spp.
	17		/		/	/	Fungus & Bacillus spp.
	A	/	/		/	/	Fungus & Bacillus spp.
None	4	/	/		/	/	
	10		/		/	/	
	1	/	/		/	/	

/ = organism identified

Table 11. Identification and enumeration of bacteria isolated from tracheal washes of ponies immediately prior to challenge with *S. zooepidemicus*.

Inoculum	Pony	Bordetella bronchiseptica	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
10-2	12	140000	2900		2000	300	20000 Bacillus
	20	800000			17000	40000	
	8	240000	2700		5000	200	
10-1	9	1200000	10000		70000	10000	
	13				1300000	2000	
	6		100	510000		5000	
Neat	14	200000					
	17	24000	10000		20000		
	A (NOT DONE)		300		130		
							"

Table 12. Mean rectal temperatures in degrees Centigrade of ponies challenged with *S. zooepidemicus*.

Inoculum	Pony	Time after challenge / hours	24	48	72	96
10-2	12	35	35	35	35	
	20	36	36.5	36	35.5	
	8	36	36.5	36.5	37	36
	Mean	36	36	36	36	
	9	36.5	36	36	36.5	
10-1	13	36.5	35.5	35.5	36.5	
	6	35.5	35.5	35.5	36	35.5
	Mean	36	36	36	36.5	
	14	36.5	37.5	36.5	35.5	
	17	37	36	35	36	36.5
Neat	A	37.5	36.5	36.5	36	
	Mean	37	37	36.5	36	
	4	37.5	36	35	35	35
	10	38	36	37	36	35.5
	1	38	36	35.5	36	36
None	Mean	38	36	35	36	35.5

Table 13. Mean breathing rates, measured as the number of breaths per minute, of ponies challenged with *S. zooepidemicus*.

Inoculum	Pony	Time after challenge / hours	24	48	72	96
10-2	12	40	44	38	34	
	20	28	36	18	16	
	8	36	42	20	18	16
	Mean	35	41	25	23	16
10-1	9	28	30	20	24	
	13	44	48	42	32	
	6	48	36	42	42	32
	Mean	40	38	35	33	32
Neat	14	44	80	40	34	
	17	62	78	44	38	32
	A	48	42	34	16	
	Mean	51	67	39	29	32
None	4	34	36	26	24	22
	10	36	36	24	26	22
	1	52	46	40	36	42
	Mean	41	39	30	29	29

Table 14. Identification and enumeration of bacteria from tracheal washes and broncho-alveolar lavage of ponies post mortem, challenged with *S. zooepidemicus*.

Inoculum	Pony	Trach. wash or BAL	Bordetella bronchiseptica	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
10-2	12	TW	2140	6	1			
		BAL	200000	50			10	
	20	TW	123600					20
		BAL	800000	70			70	
	8	TW BAL	260000					200 Bacillus
10-1	9	TW	8090000					
		BAL	6100000					
	13	TW	380000				10	
		BAL	3750000	10			10	10 Bacillus
	6	TW BAL	160000 520000		30	30	30 10	
Neat	14	TW	290					
		BAL	40				120	
	17	TW	150000					
		BAL	600	1100				140000 Micrococcus 170000 Micrococcus 190 Nocardia 360 Nocardia
	A	TW BAL	380 590		10 20			
None	4	TW	21500					
		BAL	20300		50			
	1	TW BAL	550 170		10 60			

Table 15. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from ponies challenged with 10^{10} cfu *S. zooepidemicus*.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
A	BR. LN	124		31		134	10	40 Micrococcus 21 Bacillus
	L. Left card	684						
	N. right caud			9		9		135 Nocardia 448 Nocardia
	L. Right caud			27				18 Bacillus 62 Nocardia
	L. Access lobe	>177						
14	BR. LN							42 Bacillus 11 Nocardia
	L. Access lobe	21		1548				
17	Heart blood		340					40 Bacillus
"								

KEY

Br.LN = Bronchial lymph node card = cardiac lobe

Access lobe = Accessory lobe

caud = caudal lobe

N = Tissue which is visually normal

L = Lesion

Table 16. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from ponies challenged with 10⁹ cfu *S. zooepidemicus*.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
6	BR. LN	22		88		22		22 Bacillus
	L. Left lat car	306						
	L. Right lat car	>1961		13				
	L. Access lobe							Overgrown by Bacillus
	N. car lat caud	80		36				36 Nocardia
9	BR. LN	317		1		1		
	L. Left car	345						
	L. Right car	>390		5				
	N. Right caudal	28						
13	BR. LN			3				5 Bacillus
	N. Right car	>242						
	N. Left car	>353						

KEY

Br. LN = Bronchial lymph node

Access lobe = Accessory lobe

caud = caudal lobe

N = Tissue which is visually normal

card = cardiac lobe

L = Lesion

Table 17. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from ponies challenged with 10⁸ cfu *S. zooepidemicus*.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
8	BR. LN	1380						
	N. Left lat car	>1803						
	N. Left lat car	>2750				800		
12	BR. LN	107	13	67		267	13	
	L. Right lat car							1587301 Nocardia
	N. Left lat car	40		10				10 Nocardia 20 Bacillus
20	L. Access lobe	16	16	82				13 Nocardia
	L. med car		20	42				
	N. Right caud			198				
	N. Left lat car	234		16				Overgrown by Bacillus
								78 Coliforms

KEY

Br.LN = Bronchial lymph node card = cardiac lobe
Access lobe = Accessory lobe L = Lesion
caud = caudal lobe
N = Tissue which is visually normal

Table 18. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from unchallenged ponies: *S. zooepidemicus* experiment.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
1	BR. LN		21	21				
	Ocular discharge			+			+	Bacillus Enterobacter
	N. Left card lat		16	32			8	8 Nocardia
4	N. Right card lat		22	242				22 Nocardia
	Ocular discharge			+			+	Bacillus
	N. Left card lat	>4342					>526	

KEY

Br.LN = Bronchial lymph node

card lat = cardiac lateral lobe

N = Tissue which is visually normal

L = Lesion

Table 19. Identification of bacteria isolated from nasopharyngeal swabs of ponies immediately prior to challenge with *A. equuli*.

Inoculum	Pony	Bordetella bronchiseptica	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zoepidemicus	Other
10-2	21	/	/	/	/	/	
	B	/	/		/	/	
10-1	2		/		/	/	Coliform
	5	/	/		/	/	
	15	/	/	/	/	/	
Neat	7	/	/		/	/	S. equisimilis
	11	/	/	/	/	/	
	16	/	/		/	/	
None	10	/	/		/		Beta-haemolyticStrep
	19		/	/	/	/	

/ = organism identified

Table 20. Identification and enumeration of bacteria isolated from tracheal washes of ponies immediately prior to challenge with *A. equuli*.

Inoculum	Pony	Bordetella bronchiseptica	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
10-2	21 (NOT DONE) B	49000	400			50000	3400 Pseudomonas
10-1	2	5100	20		110		
	5	140000	200		3900	2000	
	15	400000	600		50000	6000	
Neat	7 (NOT DONE) 11 16	6500000 210		350000	40000	50000 90	Bacillus
None	10 19	2000 2000		700 2500	240 3400	10 150	800 Bacillus

Table 21. mean rectal temperatures in degrees Centigrade of ponies challenged with *A. equuli*.

Inoculum	Pony	Time after challenge / hours	24	48	72	96
10-2	21	35.5	35.5	35	36.5	
	B	37	39	36.5	36.5	
	Mean	36.5	37.5	36	36.5	
	2	35	35.5	35.5	35.5	36.5
10-1	5	36	36	36	35.5	
	15	38	35.5	36	35.5	
	Mean	36.5	36	36	35.5	
	7	34.5	35.5	35.5	36	
Neat	11	36.5	36.5	36.5	36.5	36.5
	16	35	35	35.5	35.5	
	Mean	35.5	36	36	36	
	10	36	36.5	36.5	35.5	35.5
None	19	34.5	36.5	36	36	36.5
	Mean	35.5	36.5	36.5	36	36

Table 22. Breathing rates, measured as the number of breaths per minute, of ponies challenged with *A. equuli*.

Inoculum	Pony	Time after challenge / hours	24	48	72	96
10-2	21	24	30	16	36	96
	B	16	24	20	18	
	Mean	20	27	18	27	
	2	28	34	38	36	
10-1	5	30	48	46	26	32
	15	30	26	24	20	
	Mean	29	36	36	27	
	7	22	34	26	24	
Neat	11	32	46	30	44	40
	16	32	34	28	26	
	Mean	29	38	28	31	
	10	24	22	24	20	
None	19	22	36	32	18	18 30
	Mean	23	29	28	19	

Table 23. Identification and enumeration of bacteria from tracheal washes and broncho-alveolar lavage of ponies post mortem, challenged with *A. equuli*.

Inoculum	Pony	Trach. wash or BAL	Bordetella bronchiseptica	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zoepidemicus	Other
10-2	21	TW	10	10				Bacillus
		BAL		100				Acinetobacter
		TW			660			Bacillus
10-1	B	BAL	2600					
		TW	500					
		BAL	300					
		TW	400					
		BAL	1500					
10-1	15	TW	210000					
		TW	200		200			
		BAL						20 Aeromonas
Neat	7	TW	410					
		BAL left lung			1300		100	
		BAL right lung	40	10			20000	
		TW	30000				20	
		BAL	370000	180				
None	16	TW	600	240				
		BAL						30 Nocardia
								Bacillus Pseudomonas
		TW	30000					
		BAL	20000					
None	19	TW		200				
		TW				170	300	
		BAL					60	Aspergillus Bacillus

Table 24. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from ponies challenged with 10^{10} cfu *A. equuli*.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooeptidemicus	Other
7	BR. LN		360	227		213		13 Nocardia
	L. Right diag.		5	2	14	10	2	
	L. Right card			27	1036	360	27	
	N. Left diag	65	516	16		226		
11	BR. LN	81	30	131	81	475		314 Nocardia
	L. Left card				6		442	
	N. Left diag.		299		610			
16	BR. LN			31		1		314 Nocardia
	L. Left card			23				
	N. Left diag.		20	39				

KEY

Br.LN = Bronchial lymph node

L = Lesion

N = Tissue which is visually normal

diag. = diaphragmatic lobe

card = cardiac lobe

Table 25. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from ponies challenged with 10^9 cfu *A. equuli*.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zocephemicus	Other
5	Br. LN	50	120	30		10		
	N Access. lobe	1337	54					11 Nocardia spp.
	N Left Diag.		36					
15	L. Access lobe			178				
	N. Right diag.	19				38		
2	Br. LN		90	708				
	L Access lobe		36	800	18			18 Entero 18 Bacillus
	N. Left diag.	2041						

KEY

Br. LN = Bronchial lymph node

Access lobe = Accessory lobe

diag. = diaphragmatic lobe

N = Tissue which is visually normal

L = Lesion

Table 26. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from ponies challenged with 10^8 cfu *A. equuli*

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zooepidemicus	Other
B	BR. LN		88	88		876		
	L. Left DC. con.	280 + 146small				232		
	N. Left diag.	707	10	20	30	71		
21	BR. LN	71	316	245	20			10Proteus41Aeromonas
	N. Access lobe		40					10 Bacillus
	N. Left diag.	91	1545	1818		364		182 coli 91 Serratia
“								

KEY

- Br.LN = Bronchial lymph node
- DC con = Dorsal cardiac congestion
- Access lobe = Accessory lobe
- diag. = diaphragmatic lobe
- N = Tissue which is visually normal
- L = Lesion

Table 27. Identification and enumeration of bacteria expressed as per gram of tissue sampled post mortem from unchallenged ponies: *A. equuli* experiment.

Pony	Site	Bordetella bronchiseptica	Acinetobacter spp.	Staphylococcus spp.	Pasteurella spp.	Non-haemolytic streptococci	Streptococcus zoepidemicus	Other
19	BR. LN		188	50	38	688	25	13 Bacillus
	N. Access lobe		39	20		588		
	N. Left diag		26			79	18	
	TDM mucopus						>1919	
10	BR. LN		54	14		120		20 Aeromonas 28coliforms 14Serratia
	N. Left diag.	14	236	278		56		
	L. Left apical		43	587		152		
“								

KEY

Br.LN = Bronchial lymph node
L = Lesion
Access lobe = Accessory lobe
diag. = diaphragmatic lobe
apical = apical lobe

N = Tissue which is visually normal
TDM = Tracheal diptheroid membrane

7.0 CHAPTER SEVEN

DISCUSSION.

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7.2 *Actinobacillus* and *Pasteurella* species isolated from the distal trachea of horses with bacterial lower airway disease.

When the species of *Actinobacillus*/*Pasteurella* involved in equine lower airway disease were investigated, results indicated that much of this group of isolates from the lower airway of training Thoroughbred horses is accounted for by *A. equuli* and *A. suis* -like isolates, with *A. equuli* predominating. This relative importance is in agreement with Kim, Phillips and Atherton (1976), for *A. suis* -like isolates. A retrospective study identified *A. suis* -like organisms in the transtracheal aspirates and pleural fluid of horses with acute haemorrhagic pulmonary infarction and necrotizing pneumonia (Carr *et al.* 1997). This study also found that *A. suis* -like isolates, and *A. ligniersii* were associated with inflammation of the lower airway, but this should be interpreted within the limitations of the data. Previous analysis of bacteria associated with lower airway inflammation (Wood *et al.* 1993), did not differentiate between the species of *Actinobacillus* / *Pasteurella*, which is probably why *Actinobacillus* is associated with disease in some situations but not others. Vaissaire *et al.* (1988), identified predominantly *A. equuli*, but also *A. suis* and *A. ligniersii*, from a range of conditions in mares and foals, which supports their pathogenic role in the horse. Whether the association of these bacteria with lower airway disease signifies a primary pathogenic role, or forms part of a complex of agents with a secondary role remains to be determined. Given the frequency of isolation of *A. equuli*, work on it in the context of equine LAD was continued.

7.3 Utilisation and binding of equine transferrin by *Actinobacillus equuli* and characterisation of its iron regulated proteins.

Given the taxonomic relatedness and parallel pathogenic roles of *Pasteurella* / *Actinobacillus* species in horses (Wood *et al.* 1993), *A. pleuropneumoniae* in pigs (Sebunya and Sanders, 1983) and *P. haemolytica* in sheep (Gilmour, 1980), it seemed possible that *A. equuli* could produce a transferrin binding protein, which might enable utilisation of iron bound to transferrin. *A. equuli* grown in iron limited conditions bound equine holotransferrin but iron replete cells did not. Similar studies of the related bacterium *A. pleuropneumoniae* revealed that haemoglobin bound the outer lipopolysaccharide, yet this was not utilised as an iron source (Bélanger *et al.* 1995). To ensure that this artifact was not disguising the true host iron carrier protein that *A. equuli* obtains iron from, an assay was used to test whether the isolates were able to utilise iron complexed with various mammalian carrier proteins as a sole source for growth. These equine isolates utilised iron complexed with equine transferrin but not transferrin from other species, which might explain why *A. equuli* is almost exclusively a pathogen of equines.

The binding of holotransferrin by sodium salicylate extracts might suggest that the transferrin binding part of the mechanism for obtaining iron from transferrin is an outer membrane protein, or complex of proteins, which has few or no cysteine residues open to disulphide cleavage in areas which would compromise the function of the transferrin binding site. Although whole bacterial cells and sodium salicylate extracts bound transferrin, the culture supernatants did not, suggesting that these *A. equuli* isolates utilise the iron carried by equine transferrin via a direct binding mechanism. If a siderophore type mechanism exists for these isolates, it is unlikely that it would be detected by the methods employed in this study. Whole cells of isolate R grown in iron limited culture bound transferrin, but sodium salicylate extracts of the same culture did not; the growth of isolate

R was also slower than the other isolates in medium for which the available iron was equally restricted. Taken together, the evidence suggests that there is something different about the iron scavenging mechanism of isolate R and that *in vitro*, its iron scavenging mechanism is less efficient compared with that of the other isolates. Attempts to identify a transferrin binding protein by native PAGE probably failed because the protein had little net charge, or that the binding site was altered by Western transfer conditions, or that the transferrin receptor is actually a complex of proteins.

Cultures grown in iron limited conditions contained shorter cells which took up safranin counterstain more readily than cultures grown in iron replete conditions, suggesting that iron limitation could affect the morphology of the four *A. equuli* isolates. It was noted that iron limitation changed lipopolysaccharide (LPS) structure of *B. bronchiseptica* (Menozzi, Gantiez, and Locht. 1991), so it is a possibility that changes in staining behaviour could be due to this. Iron limitation also appeared to increase the viscosity of the culture supernatants, suggesting an increase in the production of a soluble capsule or slime layer. These observations, taken together, suggest that *A. equuli* may divert cell resources into capsule and / or LPS changes in conditions of iron limitation, perhaps as mechanisms to aid growth and survival within the host.

The *A. equuli* isolates produced iron regulated proteins, which were identified as those exclusively present or those with visibly increased expression in extracts of iron limited cultures. The other proteins (non-IRPs) produced by bacteria both in iron limited and normal medium were very similar in molecular mass between all four isolates.

Immunoblotting with rabbit antisera to the four isolates suggested good antigenic cross-relatedness between the non-IRPs of *A. equuli* and a spectrum of relatedness between the IRPs of different isolates. By spectrum of relatedness is meant the lack of cross-reactivity between two representative isolates of greatest dissimilarity which both share antigenic relatedness with isolates of intermediate relatedness. In molecular antigenic terms, it could be hypothesised that the IRPs of the "intermediate isolates" have different epitopes, some

shared with one of the most "dissimilar isolates" and some shared with the other. For isolates S, X and Y, there was evidence that a transferrin binding mechanism was present in the extracts with which the rabbits were immunised, so the similarity of the IRP molecular masses for the isolates may be an indication of determinants with a common function, such as transferrin binding. This may mean that a vaccine could act by preventing *A. equuli* from thriving in the host by depriving it of iron, through blocking the bacterial transferrin binding site with specific antibody, so long as the mechanism identified is the only iron scavenging mechanism employed by this bacterium. However, the natural antibody in the equine sera tested discriminated more clearly between the IRPs of the different isolates than the immune rabbit antisera. There is evidence from the study of meningococcal transferrin binding proteins that rabbit antisera are generally more cross-reactive than, for example, mouse antisera and also that both antigen preparation and administration route may affect the extent of cross-reactivity (Ala'Aldeen *et al.* 1994).

Nevertheless, the greater dissimilarity between isolates R and X detected using rabbit antisera were observed with the horse sera. The two younger groups of ponies (A and B) reacted only with IRPs in isolates X and Y which suggested either the greatest antigenic similarity between these two or that infections with strains related to both of these isolates had occurred. Since rabbit antisera revealed strong cross-reactions between IRPs of isolates Y and S and the reactivity of equine sera was to relatively few IRPs of isolate Y, the latter case seemed most probable. Consequently, the immunogenic relationship between isolates detected with rabbit antisera may hold for the equine immune system. Either the equine immune system may have responded to epitopes different from those recognised by the rabbit, or the antibodies to IRPs were produced in response to an infection by isolates of *A. equuli* which shared some, but not all, of the antigenic forms of IRP present in isolates X and Y. This might explain why there were equine antibodies to IRPs of different molecular mass in extracts of the different isolates. The greater cross-reactivity of antibody to IRPs from different isolates in the sera of older ponies might be explained by the exposure to a wider range of *A. equuli* types related to age and history of mixing with other ponies bearing more diverse isolates. Higher titres of antibody to *A.*

equuli were associated with increasing age (Nicoletti, Mahler and Scarratt, 1982), which would support this hypothesis.

If immunity can depend on antibodies neutralising the transferrin binding capacity of IRPs, then these results suggest the need for more than one strain in a vaccine. There seemed to be considerable cross-reactivity between the non-IRPs so that, if they also play some role in protective immunity, the number of isolates in a vaccine upon which broad immunity to *A. equuli* depends may be reduced. However, the general antigenic similarity of the non-IRPs of these isolates and antigenic variation of IRPs is suggestive of immune selective pressure on an essential virulence determinant to vary, so as to escape immunity arising from infections by earlier strains.

In terms of vaccine design, based upon experience with *A. pleuropneumoniae* and *P. haemolytica*, protective immunity correlates well with the presence of antibody to toxins. There is an urgent need to examine these tracheal isolates for the possession of toxins similar to the APX group of toxins and *P. haemolytica* leucotoxin.

Finally, most of the data involves Thoroughbred horses in training. It would be interesting to see whether there were any differences in the species isolated from the Thoroughbred horse and other breeds over time, and whether there is any correlation between the presence of *Pasteurella* / *Actinobacillus* species and the transferrin type. There are seven transferrin alleles in the horse, normally analysed in blood typing, some of which are strongly associated with certain breeds and may influence the ability of these species of bacteria to obtain iron and hence establish infection. If a link were found, animals could be targeted for vaccination which may be particularly susceptible.

7.4 Antigenic and opsonic relatedness of *Streptococcus zooepidemicus* isolates from the equine trachea.

S. zooepidemicus M-like proteins were recently found to be structurally different from Group A M proteins (Walker and Timoney, 1994) and were found to lack some repeats found in group A streptococci; it has been hypothesised that M proteins in group A and M-like proteins in group C streptococci have different evolutionary origins. Nevertheless, hot acid extracted proteins in this study were called M-like proteins because they are presumed to function similarly to Lancefield Group A M proteins. Indeed immunisation with hot acid extracts from Lancefield Group C streptococci stimulated opsonogenic antibody as it would for Group A streptococci.

The study revealed complex antigenic relationships between the proteins in hot acid extracts of the five *S. zooepidemicus* isolates, yet few differences in sizes of polypeptides in silver stained SDS-PAGE gels, which suggested that the proteins present were structurally similar. Rabbit antisera contained antibody to proteins in homologous and heterologous extracts. A 41KDa protein was thought to be important for protective mucosal immunity to *S. equi* in the horse (Galan and Timoney, 1985). In this study, rabbit antibody was cross-reactive to a 41KDa peptide in two of the extracts of isolates; it has been suggested that there was a homologue of the *S. zooepidemicus* M-like protein in *S. equi* (Timoney, Umbach, *et al.* 1994).

As with *A. equuli*, the cross-reactivities of antibodies in rabbit antisera suggested a spectrum of antigenic relatedness between the higher molecular mass polypeptides in hot acid extracts of the five *S. zooepidemicus* isolates tested. Generally, there was more cross reactive antibody to the lower molecular weight fragments, but it should be kept in mind that since these are polypeptides surviving hot acid, many of them are likely to be hydrolysis end products. This suggests that the whole molecule or large fragments may have more strain specific conformational epitopes which are less stable and present in only small quantities, whilst smaller fragments are likely to be the more stable linear epitopes. Also, it is important to recall that rabbits were immunised with the hot acid extracts and

the smaller, more predominant hydrolysis products may have stimulated a stronger immune response than the larger, less concentrated molecules. Isolate B was the exception to this with a number of cross reactive higher molecular weight proteins detected by antibody present in four of the antisera, perhaps indicating that the extent of hydrolysis in the different extracts were not the same. The smaller, more cross-reactive polypeptides may have been fragments of R antigen, which were shown to provoke cross-reactive antibody, but protective immune responses were not demonstrated (Timoney, 1986). These could have given an impression of closer antigenic relatedness and therefore greater expectations of the level of cross-protection between isolates than really existed.

The amount of total protein surviving hydrolysis in extracts of isolates D and E was lower than in the extracts of the other isolates despite using similar amounts of bacterial cells. This might be due to lower levels of M-like protein expression by isolates D and E, or the M-like proteins may be smaller in size than isolates A, B, or C.

Pre-immune rabbits had no antibody to protein epitopes in hot acid extracts of the five isolates. Different epitopes may be immunodominant for rabbits and equines, so that a false idea of relatedness may be gained by studying the antibodies from only one species. Antisera raised in one species have shown a different amount of cross reactivity to another, and both the method of antigen preparation and route of administration to the animal was reported to affect the resulting antisera (Ala'Aldeen *et al.* 1994). It is not known whether antibody to cross-reactive or uniquely recognised M-like protein fragments (or a mixture of the two) can provide protection in the equine.

Rabbit antisera to hot acid extracts were used to supplement naive whole rabbit blood for *in vitro* phagocytosis tests, to discover whether all of the antisera had a similar opsonising effect. There was evidence for good opsonisation of isolates B and C by all of the antisera and it seems possible that antibody to epitopes contained in the higher molecular weight fragments could have opsonised these isolates. Isolates A and E were not opsonised even by homologous antisera and D was not consistently, despite having cross reactive antibody

for the 41KDa fragment and a range of smaller polypeptides. This suggested that antibody raised to hot acid extracts in rabbits can be opsonic for some but not all heterologous isolates, which may be due to other opsonic factors related or unrelated to M-like proteins, failing to survive acid hydrolysis. Strain variation in the amount of hyaluronate capsule produced might be a possible target for further investigation.

These experiments were repeated using whole young pony blood mixed with the *S. zooepidemicus* isolates. It was hypothesised that young ponies would have been exposed to few different strains so any opsonogenic or antigenic cross-relationships would be easier to analyse. None of the five pony bloods opsonised isolate D and few antibodies to hot acid extracts were detected by immunoblotting. Isolate B was opsonised by all pony bloods and immunoblotting revealed antibody to polypeptides ranging from 52 to 26KDa (including the 46 and 41KDa polypeptides). Isolate A was opsonised to the greatest extent by the end of the experiment and this is reflected in an immunoblot showing antibody to polypeptides ranging from 37 to 48KDa (again inclusive of 46 and 41KDa polypeptides). Isolate C was not reliably phagocytosed throughout the experiment. This relates to an immunoblot where there was reactivity to fragments from 50 to 36KDa. As individual animals will have had varying titres of antibody, it was difficult to relate the results of phagocytosis assays with antibody reactions seen with immunoblotting.

Young pony sera were surveyed for reactivity to the polypeptides in hot acid extracts by immunoblotting of SDS-PAGE gels. There was reactive antibody present mostly to the higher molecular weight peptides but the relationships between the five isolates were as complex in the horse as in the rabbit.

Analysis of the 16S-23S RNA type of the five isolates showed that three were the same ribotype (eight types exist; Chanter *et al.* 1996), despite taking samples from outbreaks as spatially and temporally separated as possible. There was no obvious relationship between antigenic/opsonogenic behaviour and ribotype, or spatial and temporal distribution of the isolates, although a general observation was made about the variable numbers of peptides of

25KDa and above picked up by rabbit antisera on immunoblots. M protein genes in *S. pyogenes* are thought to be recombination hot spots, and the M-like protein genes of *S. zooepidemicus* are similar, so we would not necessarily expect a close relationship to exist.

For *S. pyogenes*, it was discovered that capsule and M protein were variably important in resistance to phagocytosis, meaning that for some isolates, capsule was the most important mechanism, whilst for others, M protein has a more significant effect. Given the complex results for *S. zooepidemicus* it is conceivable that this is the case also.

If immunity can depend on antibodies to M-like proteins, then these results together suggest the need for a number of isolates (with antigenically different M-like proteins) in a vaccine. There seemed to be reasonable cross-reactivity between the proteins in hot acid extracts of the five isolates, but the antigenic variation of M-like proteins is suggestive of immune selective pressure on an essential virulence determinant(s) to vary, so as to escape immunity following infection by other strains. There is now a need to establish if: a) vaccination with bacterins is protective against homologous challenge; b) to assess if protection is related to responses to M-like proteins and c) to determine the prevalence and significance of different M-like protein types.

7.5 Characterisation and cloning of a Group C Streptococcal hyaluronate associated protein tested in a murine *Streptococcus zooepidemicus* challenge model.

The strategy adopted was to express recombinant HAP for testing sera and for use in vaccination challenge studies, because purification of the native enzyme yielded a low concentration of product and involved a protocol which would be unsuitable for industrial scale-up. The *S. equi* strain tested had a similar DNA sequence to that described for *S. equisimilis* HAP (Lansing *et al.* 1993; described as HS). Some of the differences are bases at the third position in the codon, which due to the degeneracy of the genetic code, mostly encode the same amino acid. The conservation of the amino acid sequence suggests that HAP may fulfil similar roles in both species. Whatever its function is, efforts to express recombinant HAP suggest that it is toxic to *E. coli* and it seems possible that the major site of the polypeptide conferring toxicity is likely to involve a frame shifted region in fragment 1, which would not otherwise clone into pGEX.

Proteins of 53 and 55 KDa, of similar size to that described for *S. equisimilis* (Prehm and Mausolf, 1986), were purified from the *S. equi* culture supernatant, which is consistent with work which shows that HAP (described as "HS"), is shed into the culture medium of growing *S. equisimilis* (Mausolf *et al.* 1990). A similar protein could not be found using a cholate solubilization method of isolating HAP from the protoplast membrane (Prehm and Mausolf, 1986), although a 42 KDa hydrophobic protein, which could have been HS, was identified. As the 53 and 55 KDa proteins co-purified with HA and were still present in purified HA treated to remove non-covalently bound proteins, it is likely that they are covalently bound to HA, as some human hyaluronate associated proteins are (Prehm, 1995); disruption of the bonding by conditions known to dissociate most covalent bonds (Hough-Monroe and Milstone, 1991), was not achieved however. HPLC analysis of hyaluronidase treated Streptococcal HA, described by Prehm (1995) might confirm the nature of the bonding.

No evidence was found that the 55 KDa protein isolated from the culture supernatant of *S. equi* was immunogenic in the rabbit. The rabbit antisera raised to the 53 KDa protein and

recombinant protein (fragment 4), detected by immunoblotting and dot blots, showed that both were immunogenic in the rabbit and cross-reactive, which was reduced by absorption, confirming that the recombinant was a fragment of the 53 KDa protein, as hoped.

Part of the recombinant HAP (fragment 4) significantly delayed the time to death and number of viable bacteria in the lungs in mice challenged intraperitoneally with *S. zooepidemicus*. A similar result was obtained for challenge with *S. equi* (Chanter, personal communication). None of the other fragments had a similar effect, even though in most cases, mice had some antibody to the fragment they were immunised with. The mechanism of protection was not fully investigated, but the *in vitro* data suggested that it could involve interference of capsule production by antibody, as hypothesised. The capsule inhibition data was qualitative, but could be quantified using buoyant density centrifugation, as described by DeAngelis and Weigel, (1994), for detection of HA capsule from group A Streptococci.

HA is already on the market as an surgical ophthalmological support and other uses are being considered, so extensive safety studies have already been completed. However, antibodies were found to be covalently bound to hyaluronate in the synovial fluid of patients with rheumatoid arthritis, and it is thought likely that antibodies themselves become antigenic, leading to acute inflammation (Prehm, 1995). Similarly, antibody to the *S. pyogenes* 56 KDa HAP was found in patients with rheumatic fever, suggesting that it might cross-react with heart cells and lead to lysis (Prehm, Herrington, *et al.* 1995). There was no evidence of any problems in the rabbits, or mice immunised with HAP. However, these studies only concern short term effects, so further work must assess the risk of long term autoimmune problems. The next logical step is an equine acceptability trial, then to test the potential of recombinant HAP (fragment 4) to protect equines against experimental *S. zooepidemicus* lower airway challenge and/ or natural challenge in the field.

7.6 Equine *Streptococcus zooepidemicus* and *Actinobacillus equuli* challenge model development.

There was a histological and gross pathological dose response to challenge with *S. zooepidemicus*, but few challenge organisms were recovered from lung lesions. Immunostaining with homologous *S. zooepidemicus* rabbit antiserum however, revealed the presence of *S. zooepidemicus* antigen. PCR in situ hybridization could be employed in the future to confirm that the *S. zooepidemicus* identified in the lesions was the challenge strain.

The results were complicated by the presence of *Bordetella bronchiseptica* infection, which peaked in numbers of organisms and ponies infected at the commencement of the *S. zooepidemicus* challenge. *B. bronchiseptica* associated with an inflammation score of 1 or greater was likely to have affected the results of both challenges, as inflammation was already present. The many *S. zooepidemicus* present in tracheal washes and nasopharyngeal swabs immediately prior to *S. zooepidemicus* challenge, and immunoblotting evidence indicated that the ponies may have produced additional antibody to polypeptides in hot acid extracts of the challenge isolate by the time they arrived. It is likely that the ponies became susceptible to disease because of the journey to the Animal Health Trust which they made in the week prior to challenge. *S. zooepidemicus* pneumonia was previously associated with transport of horses (Oikawa *et al.*, 1994). Posture often adopted whilst travelling has been demonstrated to slow mucociliary transport (Raidal, Love and Bailey, 1996), and in calves, stress has been shown to diminish infiltration and production of superoxide by polymorphonuclear leucocytes (Henricks, Binkhorst and Nijkamp, 1987).

S. zooepidemicus, *Actinobacillus/Pasteurella* sp. and *B. bronchiseptica* are frequently isolated alone or in mixtures from the lower respiratory tracts of foals (Mair, 1989; Knight & Hirsh, 1982; Darien *et al.* 1990; Hirsh & Jang, 1987), and polymicrobial infections are thought to be common (Martens *et al.* 1989; Wilson, 1992; Mansmann, 1982; Knight &

Hietala, 1978); these previous observations are consistent with the findings of this current experiment.

Parasites were found, and these are associated with unthriftiness and increased susceptibility to infection (Morris, 1978; Mansmann, 1982). Large numbers of *S. zooepidemicus* have been previously reported in association with lesions in the gut mucosa caused by *Gasterophilus* spp. (Welsh, 1984), which were found in the intestines of a number of the ponies.

There was a clear gross and histopathological dose response for ponies challenged with *A. equuli* although there is some evidence from immunoblotting that there was an increased serum antibody titre to some IRPs on the day of challenge, which were not detectable before. No live bacteria were recovered from the lesions, but immunostaining revealed the presence of antigen. There is little direct relationship between events in the upper and lower respiratory tracts in either challenge and it is difficult to distinguish pathogens from normal flora in the upper respiratory tract.

For future challenge work, the use of SPF animals would be ideal, but given the practical constraints upon use of these animals, revision of methods used for non-SPF animals could be more appropriate. Non-SPF animals could be housed in containment facilities until most disease has died down, allowing a longer settling in period, then tested for immunity to challenge organisms. The screening process for naive animals could potentially fail if animals are in the early stages of infection and the reactive circulating antibody titres are low because antigen binding sites are in excess of the antibody available (Mimms et al, 1995). For example the antibody response to two IRPs in extract of *A. equuli* isolate S was not detected on the day of euthanasia although it was seen on the day of challenge. An improvement may be to test for serum antibody a number of times over a defined time period. Another useful addition to the data would be to put ponies through the same manipulations as the challenge ponies, but without the challenge organisms, since inoculation of fluid into the lungs of neonatal calves resulted in pulmonary dysfunction

(Killingsworth *et al.* 1987). Although the pathology was related to the challenge dose, it would be interesting to know the background pathology caused by the fluid. Aerosol designs would also be worth examining as a means of dispensing challenge organisms.

7.7 Conclusions and future work.

It seems as though *A. equuli* and *A. suis* are the most significant of the Actinobacillus/Pasteurella species, but it would be useful to know which pathogenic determinant(s) they possess that the other species may not, perhaps toxins. It would be useful also to be able to demonstrate inhibition of transferrin binding by antibody to iron restricted *A. equuli*, thereby demonstrating a potential mode of action for a vaccine. Attention must also be given to the adjuvant used in a potential equine vaccine, since *P. haemolytica* bacterins in oil adjuvant given to calves challenged with *P. haemolytica* had a protective effect, whereas the same in aluminium hydroxide did not. This may be a potential problem since Freund's adjuvants cannot be used in the horse. The dose given also had an effect on the efficacy in calves, so this would need careful optimisation.

It now seems unlikely that M-like proteins of *S. zooepidemicus* would be cross-protective in the horse, but the HAP approach looks more promising. First a reliable challenge model in the equine requires development. Intercurrent infections are common in the ponies used and even with efforts to isolate them, these infections developed rapidly. Also, the pathology could not be reliably attributed to the challenge organisms. This study and some of the literature suggested that the role of *B. bronchiseptica* in equine airway disease is worthy of full epidemiological investigation for potential inclusion in a vaccine. Equine *B. bronchiseptica* isolates produce a number of IRPs (Ward, In Press) so this might be a way forward.

8.0 CHAPTER EIGHT

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